Investigating the relative influence of genetic drift and natural selection in shaping patterns of population structure in Delphinids (Delphinus delphis; Tursiops spp.)

MOURA, ANDRE,EURICO,VIOLA

Investigating the relative influence of genetic drift and natural selection in shaping patterns of population structure in Delphinids (*Delphinus delphis*; *Tursiops* spp.)

André Eurico Viola de Moura

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Abstract

Speciation models relying on geographic barriers to limit gene flow gather widespread consensus, but are insufficient to explain diversification in highly mobile marine organisms. Adaptation to different environments has been suggested as an alternative driver for differentiation, particularly in cetaceans. In this study, patterns of population structure at neutral and functional markers were investigated for both common (*Delphinus delphis*) and bottlenose dolphin (*Tursiops spp.*), chosen due to high levels of morphological and ecological variation within each genus. Candidate functional markers were selected by investigating signals of positive selection in both mammals and cetaceans.

No population structure was found in the European common dolphin for neutral microsatellite *loci*, in contrast to what is observed in other sympatric cetacean species. The previously described differention of the Eastern Mediterranean Sea population, probably results from a recent human-mediated bottleneck. Functional markers showed almost complete uniformity suggesting purifying selection. One non-synonymous mutation in β-casein and the DQβ1 *locus* were exceptions, with patterns of population differentiation possibly the result of differences in local selective pressures.

Additionally, large mitogenomic sequences were used to investigate the worldwide phylogeography of several ecotypes/species within the genus *Tursiops*, with a recent biogeographical calibration point being used to calculate divergence times. Good node resolution with high statistical support was achieved, with good separation between most ecotypes in their own lineages. However, the results give no support for a monophiletic *Tursiops*. Divergence times are clustered in specific geological periods characterized by climatic fluctuations from cold to warmer periods.

The Common and bottlenose dolphins exhibit contrasting patterns of population structure in an environment containing few geographical barriers. Such difference is speculated to be related with different feeding ecologies and social structures, although data on such are still limited. Although selection can be detected in the genomes of cetaceans both at the species and population level, current patterns of differentiation are thought to occur mainly due to drift.

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Statement of Copyright

Declaration

All data presented in this thesis represents the authors own original work, save the individual contributions expressed in the acknowledgements, or sources quoted throughout the text. Chapter 3 was made in collaboration with the staff from the Centre for GeoGenetics, University of Copenhagen, who contributed with laboratory work and data processing. Their contribution is properly credited in the appropriate section of the referred chapter.

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Chapter 1 - Introduction

1.1. Differentiation in Animals

One of the key innovations brought about by Darwin into biological thinking, was that diversity in living organisms was essential for their survival in an environment where resources are limited (Darwin, 1859). The mechanisms that originate and maintain such diversity have been the focus of intense and continuous study ever since. In the extreme, two populations will split in such a way that hereditary material is no longer exchanged between them and speciation is said to occur. Although mechanisms are known to cause speciation instantaneously over the course of a single generation (e.g. Coyne, 1992; Wood *et al.*, 2009), in animals it is usually preceded by some form of population differentiation, and mechanisms behind each step will be necessarily correlated (Kondrashov, 1992). As such, research on speciation benefits greatly from insight into population differentiation, with the opposite also being true.

1.1.1. Geographic speciation

In one of the most influential works on speciation, Mayr (1963) stated that the process starts when gene flow between two populations becomes interrupted by an external geographical barrier, after which given enough time the two populations would develop reproductive isolation and gene flow would cease even if those populations regained contact. This model was named allopatric speciation, due to the geographical isolation that had to occur between the diverging populations. Predictions made by this model were found to occur in several natural populations, namely tropical birds (Mayr, 1963), and it received increased support with its replication in a laboratory environment with fruit flies (Dobzhansky & Pavlovsky, 1971; Dobzhansky, 1972). Since then, several other laboratory experiments have supported this model (Weinberg *et al.*, 1992; Rice & Hostert, 1993). Numerous cases of wild populations fitting the model have since been reported, especially after the development of phylogeography as a research field (Avise *et al.,* 1979a; Avise *et al.*, 1979b; Avise *et al.*, 1987). In one of the most well studied systems, the divergence of most European vertebrates can be traced back to the Pleistocene glacial cycles when ice caps covered most of central Europe and species were forced to three isolated refugia: Iberia, Italy and the Balkans (Taberlet *et al.*, 1998; Hewitt, 2000; Hewitt, 2001; Weiss & Ferrand, 2007).

In North America more complex patterns can be detected owing to the more complex geography of that continent (Remington, 1968; Swenson & Howard, 2004; Swenson & Howard, 2005), supporting the role of geography in the speciation process.

1.1.2. Limitations of geographical speciation

Mayr (1963) also recognized a clear limitation of this model by noting that the scarcity of geographic barriers in the open ocean, made it unclear as to how the allopatric model would apply to marine organisms. Mayr (1963) stated that sea urchins appear to fit his models, but these are animals with limited mobility and planktonic larvae, which makes them vulnerable to physical phenomena such as currents that could act as dispersal barriers in a similar fashion to geography on land (Palumbi, 1994; Jackson & Cheetham, 1999)(although note the re-evaluation of Mayr's sea urchin analysis using molecular phylogenetic data (Palumbi & Lessios, 2005)). Nevertheless, for free swimming marine organisms with high dispersal abilities, geographic barriers are unlikely to play a large role in population differentiation and speciation.

Large-scale geological phenomena have been invoked as putative geographical barriers to gene flow in marine organisms in several instances. For example, continental drift due to plate tectonics has caused the distribution of land masses to change drastically over time, and has been invoked as a mechanism to explain certain speciation events in marine organisms (Bert, 1986; Springer, 1988; Endo *et al.*, 1996). However, these large-scale processes are insufficient to explain all the diversity found in the ocean, and some authors argue that species differentiation might sometimes precede geological isolation (Knowlton & Weigt, 1998; Hurt *et al.,* 2009). Isolation by distance has also been claimed as a geographical isolating factor for speciation in the sea, although in such cases the distances involved are usually greater than or at the maximum limit of a species dispersal abilities (Kay & Palumbi, 1987; Palumbi, 1994). Yet, many marine groups will exhibit high levels of diversity, a contradiction that has been designated the "marine-speciation paradox" (Bierne *et al.*, 2003).

1.1.3. Non-geographical models of speciation

Mather (1955) first proposed that a geographical barrier was not necessary for divergence to occur, and that disruptive selection could lead to stable polymorphisms and eventually to reproductive isolation, as long as the selective pressures were strong enough relative to gene flow. This was shown to be mathematically feasible (Maynard Smith, 1962; Maynard Smith,

1966), and developments of this model have established that reproductive isolation should be preceded by race formation (Pimentel *et al.*, 1967), which is more easily achieved by habitat specific mating (i.e. individuals first choose the habitat, then mate in that same habitat (Rice, 1984)). Recent models concluded that lower levels of selection are required if a more realistic pattern of selection is assumed rather than simple disruptive selection (Artzy-Randrup & Kondrashov, 2006). Adaptation to different environments has also been shown through mathematical models to be sufficient to explain the rapid rates of speciation in adaptive radiations (Gavrilets & Vose, 2005). Several laboratory experiments in which habitat specific mating was simulated, resulted in the development of strong assortative mating between individuals with different habitat preferences, even if dispersal between them was possible (Soans *et al.*, 1974; Hurd & Eisenberg, 1975; Rice, 1985; Rice & Salt, 1988; Dodd, 1989; Rice & Salt, 1990). Studies on wild *Rhagoletis* flies found that F1 hybrids between lines of different fruit plant specialists failed to respond to either parent preferred host plant in experimental conditions (Linn *et al.*, 2004), showing that the effect was not confined to a laboratory setting.

Several cases of divergence with gene-flow have been described in the wild. A particularly well documented case is that of phytophagous insects (Bush, 1994), particularly well documented in *Rhagoletis* flies (Bush, 1969; Linn *et al.*, 2004), whose life-history adequately fit the theoretical models. Several other cases have been claimed in wild populations (Orr & Smith, 1998; Schluter, 2001; Via, 2001; Savolainen *et al.*, 2006a). However, such cases are confounded by the fact that it is usually impossible to assure that the presently diverged populations have never experienced a period of geographical isolation in the past to promote the initial differentiation (Coyne & Orr, 2004; Nosil, 2008). For example, Klicka and Zink (1997) argued that much of the diversity observed in European vertebrates began to form before the Pleistocene glaciations. Nevertheless, given the drastic geographical changes experienced in Europe, and the relatively limited data available today, invoking the glaciations almost always becomes the most parsimonious explanation. A typical example of such controversy can be seen in a discussion regarding the mechanism of speciation in insular palm trees of the genus *Howea* (Savolainen *et al.,* 2006a; Savolainen *et al.*, 2006b; Stuessy, 2006). In the marine environment, such confounding factors are less important given the scarcity of geographical barriers to dispersal, making this an ideal model to study the role of the environment in promoting population differentiation in the absence of geographical barriers.

1.1.4. Speciation in the sea

Although considerable progress has been made in the understanding of the mechanisms promoting population differentiation and speciation in the sea, it is still largely unstudied in animals whose ecology and life history are less compatible with a geographic model of speciation (Palumbi, 1994; Futuyma, 1998; Jackson & Cheetham, 1999; Mayr, 2001; Palumbi & Lessios, 2005). Marine species will often exhibit population structure on a scale largely inferior to their dispersal potential, such as cod (Ruzzante *et al.*, 1996; Hutchinson *et al.,* 2001; Pogson *et al.*, 2001; Knutsen *et al.*, 2003), herring (McPherson *et al.*, 2001; McPherson *et al.*, 2004; Jorgensen *et al.,* 2005), cuttlefish (Perez-Losada *et al.*, 2007), sea bass (Naciri *et al.*, 1999), silverside fish (Beheregaray & Sunnucks, 2001), and several species of phytoplankton (Medlin, 2007).

Oceanic currents have been suggested to limit dispersal in a similar way as geographical barriers in terrestrial environments (Palumbi, 1994; Jackson & Cheetham, 1999). This may be more likely in organisms whose dispersal is dependent on planktonic larvae, because even though these might be carried across long distances by currents, the survival capabilities of the larvae might stop them from travelling beyond certain distances (Palumbi, 1994). However, dispersal ranges are limited not only by the larval capacity to survive in the water column, but also by the ability of the larvae to find appropriate conditions to settle, meaning that ecology would be a defining parameter in addition to geography (Palumbi, 1994). In fact, even some biogeographic models that rely on Pleistocene range contractions to explain diversity patterns in marine fishes (Almada *et al.*, 2001) depend more on adaptation to local environments than on geography itself (Stefanni *et al.*, 2006; Domingues *et al.,* 2007; Domingues *et al.*, 2008). In such cases, ecological and environmental differences play an essential role in limiting dispersal and may act as effective barriers promoting speciation in a patchy environment such as the ocean. For example, in a coral reef surrounded by large patches of sandy bottom, neighborhood size (as defined in (Palumbi, 1994)) of different fish species appeared to be effectively limited by the size of the surrounding sandy patches (Chapman & Kramer, 2000).

The pattern that emerges from these data is that for marine organisms such as fishes or sessile invertebrates, it might be difficult to separate between the effects of geography and the environment in determining population differentiation. The ability of individual animals to disperse over long distances can be dependent on availability of suitable habitats, and is not necessarily correlated with gene flow over long distances (Palumbi, 1994).

1.2. CETACEANS

Cetaceans are marine mammals with high dispersal potential and no apparent geographic barriers to their movements in the ocean (Hoelzel, 2002). In spite of this, the group has experienced a rapid radiation, giving rise to a large number of species in a relatively short time (Gingerich *et al.*, 1990; Gatesy *et al.*, 1999; Gingerich *et al.*, 2001; Arnason *et al.*, 2004; Price *et al.*, 2005; Nikaido *et al.*, 2007; McGowen *et al.,* 2009; Slater *et al.*, In Press). Furthermore, several studies have shown that instead of having large panmictic populations, they can show considerable genetic and morphological structure on a regional scale (Chivers *et al.*, 2002; Hayano *et al.*, 2003; Hayano *et al.*, 2004; Krützen *et al.*, 2004; Natoli *et al.*, 2004; Karczmarski *et al.*, 2005; Natoli *et al.*, 2005; Rosa *et al.*, 2005; Sellas *et al.*, 2005; Adams & Rosel, 2006; Charlton *et al.*, 2006; Murphy *et al.*, 2006; Natoli *et al.*, 2006; Fontaine *et al.*, 2007; Gaspari *et al.*, 2007). As such, cetaceans are an interesting model to study the relative role of geography and ecology in determining population structure and speciation.

Great whales exhibit complex migration patterns that most likely determine genetic structuring and define extrinsic barriers due to fidelity to maternal migratory routes (Baker *et al.,* 1993; Palsbøll *et al.*, 1995; Palsbøll *et al.*, 1997; Baker *et al.*, 1998), and geography has necessarily been an important factor in river dolphins (Cassens *et al.,* 2000; Hamilton *et al.*, 2001; Verma *et al.*, 2004; Yan *et al.*, 2005). In a few instances, it has been speculated that variation in coastal geography and sea levels during glacial cycles might have driven the current observed patterns of variation (Fordyce & Barnes, 1994; Hayano *et al.*, 2004; Steeman *et al.*, 2009). Nevertheless, differences in local habitat have been proposed as a major mechanism promoting divergence in cetaceans (Hoelzel *et al.*, 1998; Yoshida *et al.*, 2001; Torres *et al.*, 2003; Natoli *et al.*, 2005; Sellas *et al.*, 2005; Natoli *et al.*, 2006; Bilgmann *et al.*, 2007b; Fontaine *et al.*, 2007; Mendez *et al.*, 2010). Even in river dolphins the environment appears to play an essential role. While all the river dolphins share a similar basic body morphology and behaviour (they have extremely elongated beaks, short wide pectoral fins, reduced sense of vision, and a reliance on sound to hunt their prey), the genus *Platanista* has adapted to a riverine environment independently of the other river dolphins (McGowen *et al.*, 2009). Additionally, several other selection related mechanisms have been proposed, namely: kin selection (Möller & Beheregaray, 2004; Parsons *et al.*, 2006; Gaspari *et al.*, 2007), cultural hitchhiking (Whitehead, 1998), and resource specialization (Sellas *et al.*, 2005; Hoelzel *et al.*, 2007).

Several cases of strong correlations between genetic and ecological differentiation have been reported in cetaceans (Hoelzel *et al.*, 1998; Yoshida *et al.*, 2001; Torres *et al.*, 2003; Natoli *et al.*, 2005; Sellas *et al.*, 2005; Natoli *et al.*, 2006; Segura *et al.*, 2006; Bilgmann *et al.*, 2007b; Fontaine *et al.*, 2007; Mendez *et al.*, 2010). In the harbour porpoise (*Phocoena phocoena*), for example, genetically differentiated units were geographically coincident with differences in temperature and productivity (Fontaine *et al.*, 2007), with a similar pattern being found for bottlenose dolphins (*Tursiops truncatus*) along the Mediterranean Sea (Natoli *et al.*, 2005). In Argentinean franciscana river dolphins (*Pontoporia blainvillei*) a strong correlation exists between genetic differentiation and an environmental change between a riverine and an oceanic environment (Mendez *et al.*, 2010). Also, several species seem to exhibit a differentiation between offshore and coastal populations, such as white-sided dolphins (*Lagenorhynchus obliquidens*) in Japan (Hayano *et al.*, 2004), Atlantic spotted dolphins (*Stenella frontalis*) and bottlenose dolphins (*Tursiops truncatus*) in eastern North America (Hoelzel *et al.*, 1998; Adams & Rosel, 2006).

1.2.1. Delphinids

Delphinids constitute the most diverse group of cetaceans in number of species, morphology and ecology, and they occupy a wide variety of marine ecosystems (Evans & Raga, 2001; Hoelzel, 2002). It is a monophyletic group whose origin is thought to have occurred around 10 Myrs before present (Fordyce & Barnes, 1994; Cassens *et al.,* 2000; McGowen *et al.*, 2009), whoe phylogenetic relationships have been difficult to determine. The first efforts were characterized by low resolution and a large basal polytomy (LeDuc *et al.*, 1999). A better resolution has been achieved since, but the phylogenetic position of some taxa remains difficult to determine due to inconsistent placement across different datasets (e.g. subfamily Lissodelphinae (May-Collado *et al.*, 2007)), inconsistency with classically defined taxonomic groups (e.g. the genus *Lagenorhynchus* (Harlin-Cognato & Honeycutt, 2006) and *Stenella* (May-Collado *et al.*, 2007)), and poor node support (e.g. subfamily Globicephalinae (May-Collado & Agnarsson, 2006; May-Collado *et al.*, 2007)). On the other hand, several cases of interspecific hybridization are known within delphinids (Dohl *et al.*, 1974; Spilliaert *et al.*, 1991; Miyazaki *et al.*, 1992; Heide-jorgensen & Reeves, 1993; Reyes, 1996; Baird *et al.,* 1998; Zornetzer & Duffield, 2003; Willis *et al.*, 2004; Caballero & Baker, *In Press*). Cetaceans in general have highly conserved karyotypes (Arnason, 1972; Arnason, 1974) and hybrids kept in captivity appear to be fully fertile, showing that in some cases, reproductive isolation in

cetaceans might be restricted to pre-mating barriers. Nevertheless, species that are known to hybridize will maintain their identities in the wild even when occurring in sympatry (Baird *et al.,* 1998; Caballero & Baker, *In Press*).

Delphinids are thought to have a large dispersal potential, and cases have been recorded of individuals travelling thousands of miles in just a few days (Shane *et al.,* 1986; Wells *et al.*, 1999). Together with the fact that species barriers are thought to be incomplete and recently established, large panmictic populations with wide geographical distributions would be expected to occur in dolphins. However, several cases are known which contradict this expectation. Killer whales in the eastern North Pacific exhibit marked differentiation between sympatric ecotypes specialized in different prey resources (Hoelzel *et al.*, 2007). False killer whales (*Pseudorca crassidens*) found in Hawaiian coastal waters appear to differentiate from oceanic or Central American populations, with some support for a further differentiation between Mexican and Panama populations (Chivers *et al.*, 2007). In the eastern coast of the United States significant differentiation was found between three geographically continuous coastal populations of Atlantic spotted dolphin (*Stenella frontalis*) (Adams & Rosel, 2006). In Europe, Risso's dolphins (*Grampus griseus*) show significant levels of differentiation between UK and Mediterranean samples, and evidence for further differentiation within the Mediterranean were found (Gaspari *et al.*, 2007). A study on spinner dolphins (*Stenella longirostris*) found evidence for limited connectivity between populations inhabiting different Pacific island systems, (geographic range between 16 – 1500 Km) (Oremus *et al.*, 2007). Peruvian dusky dolphin (*Lagenorhynchus obscurus*) showed evidence of limited gene flow with populations elsewhere, even though no significant genetic structure was detected between the South American, African and New Zealand populations (Cassens, 2005). These results suggest that in delphinids geography is a less important factor determining population structure, as within the same species populations spread through thousands of miles can then be differentiated across short stretches of ocean where no obvious geographic barriers are apparent.

However, few species can serve as models for assessing the role of the environment in promoting division better than the common (*Delphinus spp.*) and the bottlenose dolphin (*Tursiops spp.*). They are phylogenetically closely related, widespread cosmopolitan dolphins found in all major oceans except polar regions (Folkens *et al.*, 2002), and are characterized by an extensive morphological variation that led to the proposal of several different species within each respective genus (Hershkovitz, 1966).

1.2.2. Bottlenose dolphin (*Tursiops spp.*)

Although several different species of the bottlenose dolphin have been proposed in the past (Hershkovitz, 1966), only two species are currently recognized, *T. truncatus* widespread through all major oceans, and *T. aduncus* (the Indian bottlenose dolphin) distributed essentially through coastal areas of East Africa, Asia and northwest Australia (Folkens *et al.*, 2002). Genetic studies have shown that samples from putative *T. truncatus* and *T. aduncus* populations are consistently well differentiated (Wang *et al.*, 1999; Möller & Beheregaray, 2001; Natoli *et al.*, 2004; Charlton *et al.,* 2006; Möller *et al.*, 2008), and consistently group as independent clades in phylogenetic analyses (LeDuc *et al.*, 1999; Natoli *et al.*, 2004; Kingston *et al.*, 2009). Based on similar patterns, it was further suggested that the *aduncus* morphotypes from South Africa might represent a distinct species from Asian *aduncus* type dolphins (Natoli *et al.*, 2004), while *Tursiops* samples collected in Southern Australia were placed in their own mitochondrial lineage, separate from both *T. truncatus* and the Australasian form of *T. aduncus* (Charlton *et al.,* 2006; Möller *et al.*, 2008; Rosel *et al.*, 2009). This together with comparatively high genetic distances in comparison with *T. aduncus* and *T. truncatus* populations led the authors to propose a new species (Charlton *et al.,* 2006; Möller *et al.*, 2008). Presently, both these cases await further analysis.

Bottlenose dolphins are characterized by diverse ecology and behaviour throughout their range. Although capable of long range dispersal (dispersal events of 1500 Km for the coastal ecotype (Shane *et al.*, 1986) and 4200 Km for the offshore ecotype (Wells *et al.*, 1999) have been recorded), the coastal ecotype appears to use well defined restricted "home ranges" that can vary seasonally or be connected by specific well defined "travelling ranges" (Shane *et al.*, 1986). Diet and hunting behaviour is also extremely variable (Silber & Fertl, 1995; Smolker *et al.*, 1997; Bearzi *et al.,* 1999; Sargeant *et al.*, 2007; Sargeant & Mann, 2009), suggesting that bottlenose dolphins are capable of adapting to a variety of different ecological contexts. Social behaviour is also quite variable. A well studied population in Australia exhibits a fission-fusion like social structure, with males forming small stable alliances to herd females. These alliances can often unite in larger and more fluid superalliances (Connor *et al.,* 1992; Connor *et al.*, 1999), which have been suggested to prevent animals from dispersing too much from their native areas (Krützen *et al.*, 2004). Females, on the other hand, form small groups composed of related individuals, suggested to increase their calf rearing success (Möller & Beheregaray, 2004).

Adaptation to local conditions such as prey resources coupled with a complex social structure, might lead to habitat specific breeding and as such promote differentiation in the absence of geographical barriers (Rice, 1984; Rice & Salt, 1990). Patterns of genetic structure in the bottlenose dolphin (*Tursiops spp.*) reveal that populations from different locations around the world are generally well differentiated between each other (Tezanos-pinto *et al.*, 2009). Small-scale population structure (below recorded travelling distances (Shane *et al.,* 1986; Wells *et al.*, 1999)) has been found in many locations around the world, namely Europe (Parsons *et al.*, 2002; Natoli *et al.,* 2005; Nichols *et al.*, 2007), east coast of North America (Dowling & Brown, 1993; Hoelzel *et al.*, 1998; Natoli *et al.*, 2004; Sellas *et al.*, 2005; Rosel *et al.*, 2009), the Bahamas (Parsons *et al.*, 2006) and several locations in Australia (Krützen *et al.*, 2004; Möller & Beheregaray, 2004; Bilgmann *et al.*, 2007b). Differences in habitat use have been proposed as an explanation for the different patterns observed. Most notably, the division between "coastal" and "offshore" ecotypes described in the eastern coast of the United States is consistent with differences in morphology, diet, and habitat use (Hoelzel *et al.,* 1998; Torres *et al.*, 2003; Natoli *et al.*, 2004). Several other authors have also noted that differentiated populations appear to inhabit regions with known differences between oceanographic features (Natoli *et al.*, 2005; Sellas *et al.*, 2005; Bilgmann *et al.,* 2007b), or exhibit differences in prey choice (Dowling & Brown, 1993; Sellas *et al.*, 2005; Segura *et al.*, 2006). Other studies have further suggested that behaviour and social factors might contribute to limited dispersal. Genetic studies in Australia showed there was some level of both female and male philopatry (Krützen *et al.,* 2004; Möller & Beheregaray, 2004), with similar patterns described in the Gulf of Mexico (Sellas *et al.*, 2005), as well as in the Bahamas from photo-id studies (Durban *et al.,* 2000; Rogers *et al.*, 2004). However, although bottlenose dolphins from the Gulf of Mexico and the Bahamas (Maze-Foley & Würsig, 2002; Krützen *et al.,* 2003; Parsons *et al.*, 2003) appear to exhibit similar social structure to the one found in Australia, it is unknown if it also occurs elsewhere.

Bottlenose dolphins thus appear to have ecological and behavioural characteristics compatible with models of differentiation in the absence of external barriers to gene flow. In spite of being known to disperse over large distances, they appear to easily adapt to local ecological conditions and exhibit habitat-specific mating. However, the exact taxonomic classification within the genus *Tursiops* is still largely undetermined, and although four different species have been proposed in the literature, no single study has yet analysed the exact phylogenetic relationships between them. As such, the relative timings involved in such

differentiation events are not yet known, thus impairing any biogeographical hypothesis to be effectively validated.

1.2.3. Common dolphin (*Delphinus spp*.)

Common dolphin (*Delphinus spp*.) can be the most abundant dolphin in areas where they occur, but their distribution is now understood to be much more patchy than initially thought (Folkens *et al*., 2002; Jefferson *et al*., 2009), due to either absence of records or only limited availability of reliable information. Like the bottlenose dolphin, the common dolphin is very variable in morphology, most notably in beak length (Heyning, 1994; Murphy & Rogan, 2006; Murphy *et al*., 2006; Pinela *et al*., 2008) and colouration patterns (Heyning, 1994; Perrin *et al.,* 1995; Stockin & Visser, 2005).

Its exact alpha taxonomy is still controversial. Genetic studies made in the eastern coast of California between two well characterized morphotypes (known as the long-beaked and the short-beaked) (Heyning, 1994) showed a strong genetic differentiation between them, leading the authors to propose the existence of two species (Rosel *et al*., 1994; Kingston & Rosel, 2004). However, morphological studies in Europe and Mauritania showed that this clear morphological distinction is not necessarily found across the species range (Westgate, 2007), as several local populations exhibited intermediate (Murphy & Rogan, 2006; Murphy *et al*., 2006; Tavares *et al*., 2010) or even clinal patterns between the two morphotypes (Pinela *et al*., 2008). Genetic studies also revealed that long-beaked morphotypes found worldwide are polyphyletic within the genus, instead suggesting convergent evolution of the long-beaked morphotype in multiple locations (Natoli *et al*. 2006). A morphotype found along the coasts of India characterized by a particularly long beak has been proposed as a new species (*Delphinus tropicalis*) (Van Bree, 1971; Jefferson & Waerebeek, 2002), but further work is needed to test this hypothesis.

Little is known about common dolphin ecology, social behaviour and reproduction (Stockin *et al*., 2004). Gonadal analysis indicates that sperm competition is strong, and mating is probably promiscuous with females copulating with several males (Murphy *et al.,* 2005; Westgate & Read, 2007). Breeding has been described as seasonal (Murphy *et al*., 2005; Westgate & Read, 2007; Filby *et al.,* 2010), although in some regions calving is seen throughout the whole year (Danil & Chivers, 2006). Genetic analysis of relatedness between individuals in one mass stranding showed that this group was essentially composed of unrelated individuals (Viricel *et al*., 2008). Feeding appears to be less diverse than in the

bottlenose dolphin, focusing mainly on fish, although cephalopods and crustaceans are also frequently found in stomach contents (Pascoe, 1986; Young & Cockcroft, 1994; Ohizumi *et al.*, 1998; Silva, 1999; Meynier, 2004; De Pierrepont *et al.,* 2005; Pusineri *et al.*, 2007). The fact that common dolphins appear to feed on a large number of different species (normally pelagic or benthopelagic), with a few being very frequent, led authors to conclude that this species is an opportunistic feeder, feeding on the most abundant pelagic fish species locally (Young & Cockcroft, 1994; Ohizumi *et al.*, 1998; Silva, 1999; Meynier, 2004; De Pierrepont *et al.,* 2005; Pusineri *et al.*, 2007). Consistently, similar hunting strategies have been reported for different regions of the world (Clua & Grosvalet, 2001; Stockin *et al.*, 2009).

Compared to bottlenose dolphins, common dolphins appear to have a relatively lower level of genetic differentiation worldwide, although levels of genetic diversity are high. Differentiation was found to exist between ocean basins, and between both Atlantic coasts, although mitochondrial haplotypes can be shared across the Atlantic Ocean (Natoli *et al.*, 2006; Mirimin *et al.,* 2009). Local-scale populations are generally panmictic, although some cases of local-scale differentiation are described, namely between opposite ends of the Mediterranean Sea (Natoli *et al.*, 2008), between Tasmania and South Australia (Bilgmann *et al.*, 2008), and along the west coast of North America (Chivers *et al.*, 2005). Differences in habitat use have also been invoked as an explanation for the genetic structure found in common dolphins, most notably, to explain the separation between long-beaked and short-beaked morphotypes (Natoli *et al.*, 2006). Also, a correlation between a cline in skull length (ranging from long-beak to short-beak) and differences in isotope signatures were found in Mauritania (Pinela *et al.*, 2008), with differences in carbon signatures between two sampling locations in the Gulf of California further suggesting the existence of location-specific feeding (Nino-Torres *et al.*, 2006).

1.2.4. Environmental variability in Europe and the Iberian coast

The European coastline has several characteristics that make it an interesting place to investigate the role of ecological differences in defining population structure. Being surrounded by four very different water masses (Black Sea, Mediterranean Sea, North Atlantic and North Sea), it exhibits a strong environmental cline along its coastline between two very different biogeographic regions, the Mediterranean in the south and Eurosiberian in the north (Alcaraz *et al.*, 2006). The main transition between these two regions occurs along the Iberian coast, which is thus characterized by a considerable environmental heterogeneity (Alcaraz *et*

al., 2006). Common (*Delphinus delphis*) and bottlenose dolphins (*Tursiops truncatus*) are abundant throughout the European coast, but have differing distribution patterns and levels of population structure.

Although the bottlenose dolphin (*Tursiops truncatus*) can be found throughout the European coast, it is known to be more common in particular regions, such as several locations in the UK (Hastie *et al.*, 2004), the Sado Estuary in Portugal (dos Santos *et al.*, 2007), or the Amvrakikos Gulf in western Greece (Bearzi *et al.*, 2008). Its genetic structure is well characterized, and it has been shown to have significant structure even on a local scale. Natoli and collaborators (2005) found significant differentiation along the European coast, with genetic breaks occurring in similar regions as other marine species, suggesting a role of the environment on such structuring (Quesada *et al.*, 1995; Borsa *et al.*, 1997; Chikhi *et al.*, 1997; Roldan *et al.*, 1998; Garcia-Martinez *et al.*, 1999; Valsecchi *et al.*, 2004; Gaspari *et al.*, 2007; Abaunza *et al.,* 2008; Perez-Portela & Turon, 2008; Fontaine *et al.*, 2010). However, the differentiation between the Black Sea and the East Mediterranean can provide useful information and warrants further research. The Black Sea is characterized by having very different environmental characteristics relative to the adjacent Mediterranean Sea (Özsoy & Ünlüata, 1997), and bottlenose dolphins from this area are particularly well differentiated from East Mediterranean dolphins (Natoli *et al.,* 2005; Viaud-Martinez *et al.*, 2008). In addition, morphological differences have led to the classification of the Black Sea bottlenose dolphin as a distinct sub-species, *T. truncatus ponticus* (Viaud-Martinez *et al.*, 2008; Committee on Taxonomy, 2009). As the geological history of the Black Sea is relatively well described (Kerey *et al.*, 2004), the differentiation timeframe of its bottlenose dolphin population can be robustly estimated and used as a calibration point to further investigate other bottlenose dolphin ecotypes/species.

The common dolphin (*Delphinus delphis*) has a more continuous coastal distribution, but it is notably absent from some areas. Although extremely common along the Iberian coast, it is a relatively rare sighting on the east coast of the UK, with bottlenose and white-beaked dolphins being much more common (Evans & Hammond, 2004). In the Mediterranean Sea, although once very abundant it is now a rare sighting, having suffered a well described abundance reduction in recent years (Bearzi *et al.,* 2003; Bearzi *et al.*, 2006; Bearzi *et al.*, 2008). Morphology appears to be quite variable, with the Black Sea common dolphins being markedly smaller than other European populations, while British and Dutch dolphins appear to be larger, while Iberian dolphins show intermediate characteristics between short-beaked

and long-beaked morphotypes (Murphy & Rogan, 2006; Murphy *et al.*, 2006). Genetic studies show that diversity is high along the coast, but genetic structure is either low or altogether absent (Natoli *et al.*, 2006; Amaral *et al.*, 2007; Natoli *et al.* 2008; Mirimin *et al.,* 2009). Preliminary studies suggest a differentiation between Greece and other populations in Europe (Natoli *et al.*, 2008), while other studies suggested the occurrence of fine scale population structuring along the Iberian coast (Amaral *et al.*, 2007). However, no detailed studies encompassing the entire European coastline have been made to present, and it is still unclear what is causing the observed patterns. Given that the environmental transition occurs mainly along the Iberian coast, and that this appears to be the region where the common dolphin is more abundant and where local scale structure has been suggested, a more detailed sampling scheme from that region is required.

1.3. Analysis of Functional Diversity

Most of the above mentioned studies on population structure in wild populations have focused on markers that are likely to be neutral (such as microsatellites or mtDNA control region). However, a lack of differentiation in neutral markers does not necessarily imply a lack of adaptive divergence (Thibert-Plante & Hendry, 2009; Thibert-Plante & Hendry, 2010). There is strong support for the notion that neutral *loci* more easily cross the barriers separating diverging groups as compared with *loci* under selection (Wu, 2001). In a case of isolation caused by differential adaption (unrelated to reproductive potential), differentiation in effectively neutral markers would thus take longer to establish because of remaining levels of gene flow (Thibert-Plante & Hendry, 2010; Wu, 2001). A notable exception occurs when a particular marker is located close to a functional gene which is itself under selection. In such cases, recombination between the neutral marker and the functional one would be reduced due to the physical proximity, and this genetic hitchhiking with the functional gene would result in linkage disequilibrium (Barton, 2000; Andolfatto, 2001).

Several studies on functional nuclear markers have found a correspondence between population structure in those markers and environmental characteristics. Notable examples are the diversity in antigen binding genes of the major histocompatibility complex (MHC) and parasite load in several species (Sanjayan *et al.*, 1996; Hughes & Yeager, 1998; Janeway *et al.*, 2001; Vassilakos *et al.*, 2009), diversity of milk proteins in different cattle breeds and artificial selection by lactose tolerant human populations (Beja-Pereira *et al.*, 2003), or allele frequency distribution in hemoglobin genes depending on altitude for several mammalian species (Storz,

2007). Population genomics studies, where genomewide variation is compared across different wild populations and/or closely related species, have revealed similar patterns. For example, a comparison between closely related species in the genus *Drosophila* revealed that divergence is elevated in regions of the genome where functional genes are located (Begun *et al.*, 2007). Another study comparing divergence between independent freshwater and oceanic populations of the threespine stickleback (*Gasterosteos aculeatus*), revealed that higher divergence was observed in the same functional regions in all freshwater populations, suggested to indicate accelerated evolution in such genes due to adaptation to the freshwater environment (Hohenlohe *et al.*, 2010).

Very few studies have investigated population structure in candidate functional genes other than MHC in cetaceans. The transition from land to sea imposed drastic selective pressures that have undoubtedly left their mark in the genome of cetaceans. On a species and population scale, if the adaptation to different environments is promoting population subdivision, then such pressures should be detectable in the genome, particularly in genes with a physiologically relevant function. Under neutrality, the rate of mutations which cause changes in the amino acid structure of a protein (non-synonymous) would tend to be the same or lower than the rate of mutations that have no effect on the phenotype (synonymous) (Li *et al.*, 1985). As such, if the rate of non-synonymous substitutions is higher than the rate of synonymous substitutions in a given gene, it is considered as an indication of a positive selective pressure on that gene (Hill & Hastie, 1987; Hughes & Nei, 1988). Several genes have been found to be under positive selection using the dN/dS test (Endo *et al.,* 1996; Ford, 2002).

1.4. OBJECTIVES

This study aims at investigating the mechanisms promoting population division and speciation in cetaceans, highly mobile organisms with little barriers to dispersal. Focus will be on the common (*Delphinus delphis*) and bottlenose dolphin (*Tursiops spp.*), species with high levels of morphological variation and several species suggested within each genus. It is hypothesized that if population structure is being promoted by differences in habitat choice, then a good correspondence should be found between environmental differences and differentiation patterns in neutral markers. To test this, local-scale population structure in the European common dolphin will be assessed in chapter 2 using a detailed sampling scheme and high resolution individual-based methods, later related with known environmental differences. This species was chosen due to previous suggestions of population structure

related to environmental differences found along the European coast. If population differentiation is promoted by environmental adaptation, then specific population structure patterns are also expected in functional markers. Population structure will thus be further assessed in chapter 3 using functionally relevant nuclear markers, which will be selected by investigating signals of positive selection in cetaceans for a set of well described candidate genes. Finally, if environmental differences are a general mechanism promoting differentiation in cetaceans, the divergence times between well established extant species and ecotypes should be coincident with periods of environmental changes. For this purpose, phylogenetic relationships within the genus *Tursiops* will be determined in chapter 4 using whole mitochondrial genome sequences. Several well differentiated species/ecotypes have been described within this genus, whose divergence has been hypothesized as the adaptation to different habitats. Divergence times will be calculated between these described species and ecotypes, and checked for correlations with known ecological and geological phenomena.

Chapter 2 – Population Structure of Common Dolphin (*Delphinus delphis*) in Europe Based on Individual Microsatellite Genotypes.

2.1. INTRODUCTION

The common dolphin is a widespread species with a high degree of morphological variation (Folkens *et al.*, 2002), whose exact alpha taxonomy is still controversial (Rosel *et al.*, 1994; Kingston & Rosel, 2004; Natoli *et al.*, 2006). Along the eastern coast of California two well characterized morphotypes (Heyning & Perrin, 1994) were found to be genetically well differentiated (Rosel *et al.*, 1994; Kingston & Rosel, 2004). They exhibit several morphological differences (e.g. body length, tooth count, colouration) but the most distinctive characteristic is a difference in zygomatic-width/length-of-rostrum ratio (Heyning & Perrin, 1994). These morphotypes were thus called collectively the short-beaked and the long-beaked form, and species status was proposed for each of them (Heyning & Perrin, 1994; Rosel *et al.*, 1994; Kingston & Rosel, 2004). It is commonly accepted in the literature that these morphotypes represent different worldwide distributed species, the long-beaked common dolphin (*D. capensis*) and the short-beaked common dolphin (*D. delphis*), that have distinct distributional patterns (Evans & Raga, 2001; Folkens *et al.*, 2002; Committee on Taxonomy, 2009). However, the clear differentiation between the long-beaked and the short-beaked morphotypes is not found across the genus range (Bell *et al.,* 2002; Murphy *et al.*, 2006; Pinela *et al.*, 2008). In Europe, where morphological variation has been relatively well studied, no clear morphological distinction can be found, even though morphological characters typical of both morphotypes can be found across the range (Murphy & Rogan, 2006; Murphy *et al.*, 2006). For example, Iberian common dolphins exhibit intermediate characteristics between the short -beaked and the long-beaked morphotypes (Murphy *et al.*, 2006).

Common dolphin distribution and abundance in Europe is also not straightforward. It is the most abundant cetacean species in the Iberian coast (Sequeira *et al.*, 1996), the Gulf of Biscay and the English Channel (Brereton *et al.*, 2005), but on the East coast of the UK it is a relatively rare sighting, with white-beaked dolphins (*Lagenorhynchus albirostris*) being much more common (Evans & Hammond, 2004). In the Mediterranean Sea, although once continuously distributed and very abundant, it has suffered a drastic decline in recent decades making it extremely rare with a patchy distribution (Bearzi *et al.,* 2003; Bearzi *et al.*, 2006).

The reduction was considered to be so extreme that this particular population was listed as endangered under the IUCN Red List (Bearzi, 2003). Although traditionally represented as having a distribution roughly similar to the bottlenose dolphin (*Tursiops truncatus*), it is now increasingly represented as having a more coastal distribution, although it also occurs in oceanic environments like the Azores or Madeira archipelagos (Freitas *et al.*, 2004; Cabral *et al.,* 2005).

Genetic structure in Europe has been shown to be less marked than in the eastern north Pacific. Significant differences in allele frequencies can generally be found between opposite sides of the Atlantic, even though both share mtDNA haplotypes (Natoli *et al.*, 2006; Mirimin *et al.,* 2009). Within Europe, differentiation is weaker but genetic diversity is usually high (Natoli *et al.*, 2006; Amaral *et al.,* 2007; Mirimin *et al.*, 2009). Some evidence of structure was found within the Mediterranean Sea (Natoli *et al.*, 2008), while signs of a recent expansion were found in the eastern Atlantic (Amaral *et al.,* 2007; Natoli *et al.*, 2008; Mirimin *et al.*, 2009). Nevertheless, morphological studies show some segregation between different regions in Europe. Notably, skull size ranges of Portuguese and Irish samples are larger than those found in Spanish and British samples (Murphy *et al.*, 2006). Also, sex differences in Fst led previous authors to suggest the existence of local-scale population structure (Amaral *et al.*, 2007) that might be masked due low resolution resulting from low sample size and insufficient markers analysed.

Other cetacean species exhibit patterns of population structure along the European coastline. In the harbour porpoise (*Phocoena phocoena*), genetic structure was found between different areas in northern Europe, although high levels of gene flow could be detected (Andersen *et al.,* 1997; Walton, 1997; Wang & Berggren, 1997; Andersen *et al.*, 2001). A similar level of structure can also be detected across its entire range in Europe, with genetic breaks being particularly strong between Western Europe and Northern Africa, between the Black Sea and the Atlantic coast, and between Iberia and the North Atlantic (Rosel *et al.*, 2003; Tolley & Rosel, 2006; Fontaine *et al.,* 2007; Viaud-Martínez *et al.*, 2007; Fontaine *et al.*, 2010). White-beaked dolphins (*Lagenorhynchus albirostris*) also show a separation between southern North Sea and north of Norway (Banguera-Hinestroza *et al.*, 2010), while the sperm whale (*Physeter macrocephalus*), fin whale (*Balaenoptera physalus*), Cuvier's beaked whale (*Ziphius cavirostris*) and Risso's dolphin (*Grampus griseus*) appear to be differentiated between the Mediterranean Sea and the Atlantic Ocean (Bérubé *et al.,* 1998; Dalebout *et al.*, 2005; Engelhaupt *et al.*, 2009; Gaspari *et al.*, 2007). In the striped dolphin (*Stenella*

coeruleoalba) population structuring can be found between Atlantic and Mediterranean populations (Garcia-Martinez *et al.,* 1995; Garcia-Martinez *et al.*, 1999; Valsecchi *et al.*, 2004), but also between Eastern and Western Mediterranean, once a more intensive sampling scheme together with higher resolution genetic methods were used (Gaspari *et al.*, 2007). A similar pattern exists in the bottlenose dolphin (*Tursiops truncatus*), where strong differentiation was found between Black Sea, Eastern Mediterranean, Western Mediterranean, Atlantic and North Sea (Natoli *et al.,* 2004; Natoli *et al.*, 2005; Viaud-Martinez *et al.*, 2008). Each of these species shows stronger evidence of population structure than seen for the common dolphin over a similar geographic range (Natoli *et al.* 2006, Mirimin *et al.* 2009).

Several authors have noted that many of the genetic breaks found in cetaceans along the European coast are consistent with well described environmental breaks, and have thus suggested that differences in ecology are promoting population structure in these animals (Natoli *et al.*, 2005; Fontaine *et al.,* 2007; Gaspari *et al.*, 2007; Natoli *et al.*, 2008; Fontaine *et al.*, 2010). However, for white-beaked dolphins, due to evidence of population expansion, authors have suggested that migration into areas that became available after the glaciations was most likely responsible for patterns observed in the North Sea (Banguera-Hinestroza *et al.*, 2010). A similar process was proposed to have caused the differentiation of UK Risso's dolphins from the Mediterranean Sea, given that the UK represents the limit of the species distribution (Gaspari *et al.*, 2007). It has also been suggested that cases of local-scale structuring in common dolphin might result from founder events from larger populations, followed by adaptation to local conditions (Natoli *et al.*, 2006). A similar process was invoked in (Mirimin *et al.*, 2009) to explain the differentiation found within the Mediterranean Sea (Natoli *et al.*, 2008). It was argued that the observed decline in the Mediterranean common dolphin and consequent fragmentation might be responsible for the higher levels of structure found there (Mirimin *et al.*, 2009). Further to this point, population structure might be caused by differences in inbreeding rates that might result from, for example, differences in social structure (Sugg *et al.*, 1996; Storz, 1999; Gao *et al.,* 2007), though there are no data about this for common dolphins in the Mediterranean.

In this study, we intend to carry out a comprehensive genetic study of common dolphin in Europe, to investigate a potential correspondence between environmental breaks and genetic structure in this species. Although previous studies have investigated the species population structure in Europe, no single study has focused on the whole distributional range of the

species. This study will build upon previous efforts by adding more detailed sampling and focusing on genetic methods that increase the power to detect subtle patterns of population differentiation, if they exist. This study will also focus on the previously described differentiation of Eastern Mediterranean dolphins and investigate different hypothesis suggested to have promoted such division. Kinship-based analysis will be done to investigate differences in social structure, while a simulation based approach will be used to compare different evolutionary scenarios.

2.2. METHODS

2.2.1. Sample Collection

Samples from both stranded and bycaught animals were obtained together with biopsies from free ranging animals. Samples were obtained from several regions representing most of the European range of the species, namely Scotland, Ireland, England, Galicia, 4 locations in the Portuguese west coast, 2 locations from the Portuguese south coast, Madeira, Gibraltar, Ligurian Sea and two locations in Greece (Kalamos and Korinthiakos Gulf) (Figure 2.1, page 34).

For the Portuguese coast in particular, the availability of biopsies is extremely important, in light of other species that show differentiation between the Iberian coast and the North Sea (Natoli *et al.*, 2004; Natoli *et al.*, 2005; Fontaine *et al.,* 2007). Given that strandings are known to occur in the Iberian coast of species only found in the North Sea (Sequeira *et al.*, 1996), the reliability on a limited number of stranding samples might mask subtle genetic differences between those areas. Samples from the Portuguese coast were obtained specifically for this study, while samples from other locations in Europe were already available from other sources. Samples from Scotland, England, Galicia, Madeira, Gibraltar and Kalamos (Greece) were used previously in (Natoli *et al.,* 2006; Natoli *et al.*, 2008), while Irish samples were used previously in (Mirimin *et al.*, 2009). Samples from the Ligurian Sea, Gulf of Biscay and Korinthiakos Gulf (Greece) were obtained from stranded animals and provided by Georgios Gkafas from Durham University.

Because this study is part of an attempt to investigate the effect of the environment in shaping genetic variation, sampling also focused on the Atlantic Iberian coast, which is the area where the transition between the Mediterranean Sea and the North Atlantic Ocean is more pronounced. This involves differences not only in climate patterns, but also

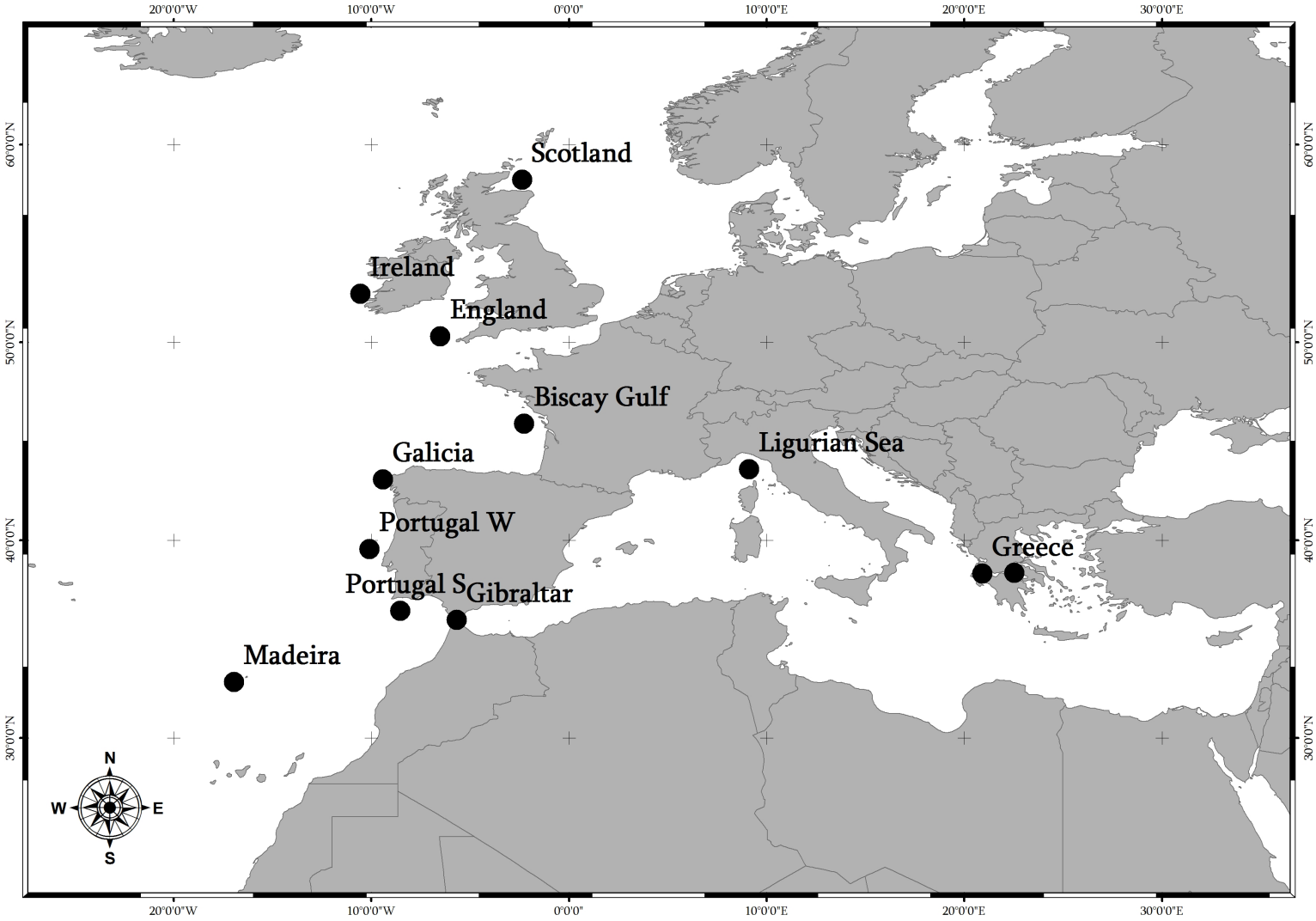


Figure 2.1. Geographical location of samples used in this study. Each dot represents a mean geographical location of all samples from a given region. Both dots in Greece represent the locations of Kalamos (Ionian Sea; left dot) and Korinthiakos Gulf (right dot).

oceanographic characteristics with, for example, the Mediterranean water being warmer and more saline (Pinet, 2003). This creates a well described transition between two distinct biogeographic regions on terrestrial biological communities, the Mediterranean and the Eurosiberian (Alcaraz *et al.*, 2006). Samples from Gibraltar and Galicia were already available, but detailed sampling from the Portuguese coast was lacking. Biopsy samples from the coast of Portugal were obtained using two different methods: a long pole with a custom made removable metal tip, loosely based on the design described in (Bilgmann *et al.*, 2007a); and a veterinary air rifle firing biopsy collection darts. Both air rifle and biopsy darts were obtained from the company Pneudart Inc. (www.Pneudart.com): rifle model 176B with floating aluminium darts with a tip length of 1.5 cm. This particular model was chosen because of its low power and the ability to regulate firing pressure. The air rifle was fitted with a fishing rod attached to the tip of the nozzle to allow recovery of the dart and to prevent it from remaining attached to the animal's skin. Sampling was carried out following the established protocol to minimize disturbance to the animals (Hoelzel, 1991; IWC, 1991), and under a permit from the Portuguese "Instituto para a Conservação da Natureza e Biodiversidade" (ICNB). Biopsies were collected from 6 locations spread along the coast (Figure 2.2, page 36), and each group of dolphins was sampled as inclusively as possible.

2.2.2. Laboratory Procedures

DNA was extracted using a standard phenol-chloroform protocol (Hoelzel, 1998). All individuals were screened for 18 microsatellite *loci* (Table 2.1, page 37) using a fraction of the forward primer labelled with a fluorescent dye (Table 2.1, page 37), and genotyped on an ABI 3130 automated sequencer. Three different fluorescent dyes were used, carboxyfluorescein (FAM), and hexachlorofluorescein phosphoramidite (HEX) and ABI proprietary NED. Amplification was made using two multiplex reactions: one amplifying 6 microsatellites, designated Set A (Table 2.1, page 37); the other 12 microsatellites, designated Set B (Table 2.1, page 37).

Amplification was done using Qiagen Multiplex PCR kit (catalog number 206143) following the manufacturers instructions. PCR conditions were optimized as follow: initial denaturation at 95°C for 15 minutes, 40 cycles consisting of annealing at 50°C (Set A)/57ºC (Set B) for 90 seconds, followed by extension at 72°C for 1 minute, followed by denaturation at 94°C for 30 seconds. Finally, one annealing step as above was followed by a final extension at 60°C for 30 minutes (Table 2.2, page 37).

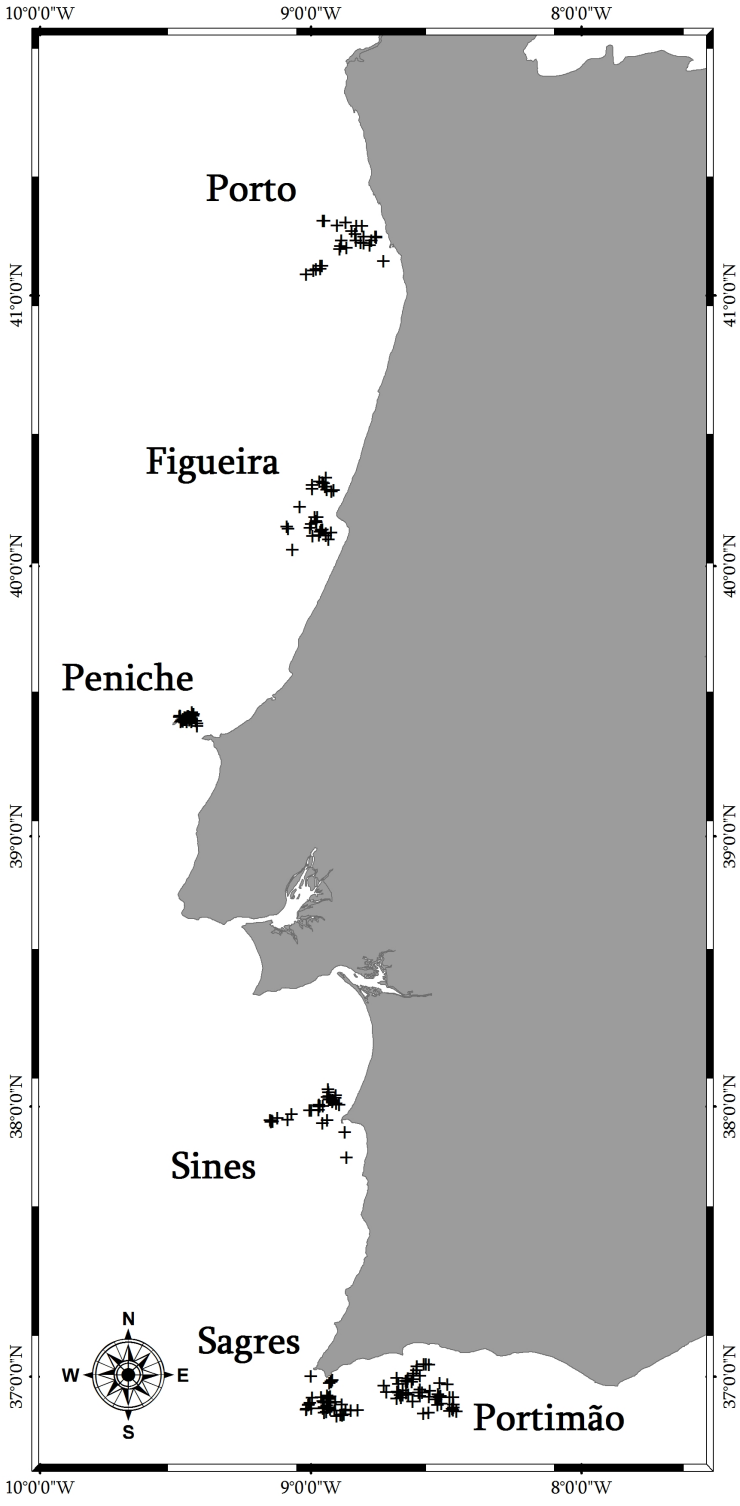


Figure 2.2. Geographical distribution of common dolphin biopsy samples collected along the Portuguese coast.

Table 2.1. List of microsatellite *loci* used in this study, grouped according to the Multiplex reactions used to amplify them. Proportion of the forward primer that was replaced by the labelled primer indicated after the Fluorescent Dye name. Allele range as described for European common dolphin in (Coughlan *et al*., 2006; Natoli *et al*, 2008). References indicate the study where they were first described.

	Locus Name	Fluorescent Dye	Allele Range	Reference
Set A	DO8	FAM-10%	88-122	(Shinohara *et al.*, 1997)
	KWM2a	FAM-20%	135-167	(Hoelzel *et al.*, 1998)
	KWM2b	HEX-10%	170-180	(Hoelzel *et al.*, 2002)
	KWM12a	NED-20%	157-178	(Hoelzel *et al.*, 1998)
	TexVet5	FAM-10%	180-214	(Rooney *et al.*, 1999)
	KWM1b	HEX-10%	181-189	(Hoelzel *et al.*, 2002)
Set B	TtruAAT44	HEX-5%	92	(Caldwell *et al.*, 2002)
	EV14Pm	NED-20%	123-159	(Valsecchi & Amos, 1996)
	TexVet9	FAM-5%	124	(Rooney *et al.*, 1999)
	Dde70	HEX-10%	133-161	(Coughlan *et al.*, 2006)
	Dde84	FAM-10%	148-166	(Coughlan *et al.*, 2006)
	Dde65	FAM-10%	184-208	(Coughlan *et al.*, 2006)
	EV37Mn	NED-20%	176-240	(Valsecchi & Amos, 1996)
	Dde69	HEX-15%	198-218	(Coughlan *et al.*, 2006)
	Dde09	FAM-15%	221-245	(Coughlan *et al.*, 2006)
	Dde72	HEX-15%	231-271	(Coughlan *et al.*, 2006)
	Dde66	FAM-20%	346-362	(Coughlan *et al.*, 2006)
	Dde59	HEX-30%	384-432	(Coughlan *et al.*, 2006)

Table 2.2. General PCR conditions used to amplify the microsatellite multiplexes used in this study

Step	T (ºC)	Time	Cycles
Denaturing	95	15'	1
Annealing	50/57	90"	
Extension	72	1'	40
Denaturing	94	30"	
Annealing	50/57	90"	1
Extension	60	30'	1

2.2.3. Data Analysis

The presence of null alleles was assessed using the software MICRO-CHECKER (Van Oosterhout *et al.*, 2004). A preliminary analysis using only the Portuguese samples gave high probability of null alleles for 3 different *loci*: TexVet5, EV37Mn and EV14Pm. Repeats of a subset of the samples revealed that all these 3 markers would result in inconsistent scoring between repeats. Also, the same result was obtained when samples from other locations in Europe were included. As such, those 3 *loci* were excluded from further analysis.

GENALEX (Peakall & Smouse, 2006) was used to identify matching genotypes based on 15 microsatellite *loci*. All duplicate samples were removed from the analysis, as were samples that only differed in up to 2 *loci*, meaning 8 samples were removed from Portugal and 3 from Kalamos (Greece). This resulted in 492 individuals from 15 different locations along the European coast (Table 2.3, this page), analyzed for 15 microsatellite *loci*.

Table 2.3. Number of samples analysed in this study, divided by each location

Location	Number of Samples
Scotland	62
Ireland	105
England	13
Biscay Gulf	29
Galicia	19
Porto	26
Figueira	26
Peniche	30
Sines	31
Sagres	40
Portimão	52
Madeira	16
Gibraltar	17
Ligurian Sea	4
Greece	22

Population structure was analysed using both classic population genetic methods and individual based methods. Due to the difference in sample numbers between locations, a possible sampling bias was assessed by plotting sample number by allele number for each population using the software ADEGENET (Jombart, 2008). Pairwise Fst was calculated between sampling locations using the software MICROSATELLITEANALYSER (MSA) (Dieringer & Schlötterer, 2003). Significance of the Fst values was calculated by individual permutation

between locations with 1,000 permutations and application of Bonferroni correction. Population structure was also tested using G-statistics as implemented in the software HIERFSTAT (Goudet, 2005). Both Principal Component Analysis (PCA) and Correspondence Analysis (CA) were performed using ADEGENET (Jombart, 2008). Factorial Correspondence Analysis (FCA) was performed on populations using the software GENETIX (Belkhir *et al.*, 2004).

Individual based clustering without *a priori* information on sampling location was done using 3 distinct algorithms. STRUCTURE and BAPS employ different Bayesian clustering algorithms that look for the number of individual groups that minimizes deviations from Hardy-Weinberg equilibrium in each group (Pritchard *et al.*, 2000; Corander, 2006; Corander *et al.,* 2008). Because both algorithms tend to give biologically implausible results when Fst values become lower than 0.1 (Corander, 2006; Latch *et al.*, 2006), they were used as independent confirmation for any patterns obtained. Additionally, patterns of population structure can be caused by historical limitation of gene flow, but also by location specific patterns of inbreeding (Gao *et al.*, 2007). The INSTRUCT algorithm accounts for such scenarios, and is reportedly more accurate in defining population structure in such cases (Gao *et al.*, 2007). Using BAPS (Corander, 2006), 4 independent runs were carried out for K≤ 2, 3, 4, 5, 6, 7, 8, 9, 11, 13, 15, where K is the maximum number of delimited groups. Such a procedure is suggested by the authors to avoid the algorithm being stuck in local optima, and as such better identify the overall optimum. STRUCTURE (Pritchard *et al.*, 2000) was run for 1 x 10^7 iterations after 1 x 10^6 burn-in for K values between 1 and 4, with 4 independent runs. The admixture model with correlated allele frequencies was used. INSTRUCT (Gao *et al.*, 2007) was run with 5 simultaneous chains for a total of 2 x 10^6 iterations with 1 x 10^6 burn-in. The joint inference of population selfing rates and population substructure was done, with K set from 1 to 10. A uniform distribution was assumed as prior for the selfing rates, and convergence among chains was assessed using the Gelman-Rubin statistic.

Isolation by distance was tested using both a Mantel Test and a Spatial Autocorrelation. Exact coordinates were available for most samples except Scotland, Gibraltar and Greece. In Scotland, the county of provenance for the sample was available, and as such a point located in the geographical middle of each county was chosen to represent the geographic location of such samples. For Gibraltar and Greece, no exact coordinates were available, but because all these samples refer to a very restricted geographic region, a single middle point was assumed to represent all the samples. Given these are also the geographically most distant samples, the

small variation between sample relative to the distance each point would have to the other European locations would most likely be negligible, and not have an effect on the overall result. Both analysis were carried out using the software ALLELES IN SPACE (Miller, 2005).

Genetic diversity was compared between the identified clusters using several diversity indices. Actual and effective number of alleles, observed, expected and unbiased expected heterozygosity were calculated using GENALEX (Peakall & Smouse, 2006). The Shannon's information index accounts for differences in sampling number, and was calculated using GENALEX (Peakall & Smouse, 2006). To check for potential differences in inbreeding, Fis was calculated in GENALEX (Peakall & Smouse, 2006). Effective population size was calculated with the software NEESTIMATOR (Ovenden *et al.*, 2007) using the point estimation method of (Hill, 1981).

As a final test for the effect of inbreeding on the obtained patterns of genetic structure, the software KINGROUP (Konovalov *et al.*, 2004) was used to assess differences in kinship within locations. Groups of individuals related at the level of parent-offsprings, full-sibs, half-sibs and cousins were determined against all the individuals. After the groups in each kinship class (parent-offsprings, full-sibs, half-sibs and cousins) were determined, the number of different groups found in each location was counted. By comparing this number to the total number of individuals, it is possible to get a notion of how much more related the individuals within a location are relative to individuals in other locations. This proportion was simply calculated in each location as follows:

$$1-(Kc/N)$$

in which **Kc** = number of groups in each kinship class; **N** = number of samples.

Severe bottlenecks are known to cause patterns similar to population structure (England *et al.*, 2003), and as such, the software BOTTLENECK (Cornuet & Luikart, 1996) was used to test for signals of bottleneck in populations found to be differentiated. Significance tests were done for all mutation models with 1,000 iterations, and with 70% proportion of single mutations for the Two Phased Model (TPM). A test for mode shift in allele frequencies was also carried out. Such a scenario was further tested by using Approximate Bayesian Computation (ABC), as implemented in the software package DIYABC (Cornuet *et al.*, 2008). In ABC methods, several competing population scenarios are modelled by simulating several different datasets fitting each defined scenario. Statistic tests are then used to assess which scenario better fits the observed data. For the present study, 4 different scenarios were tested

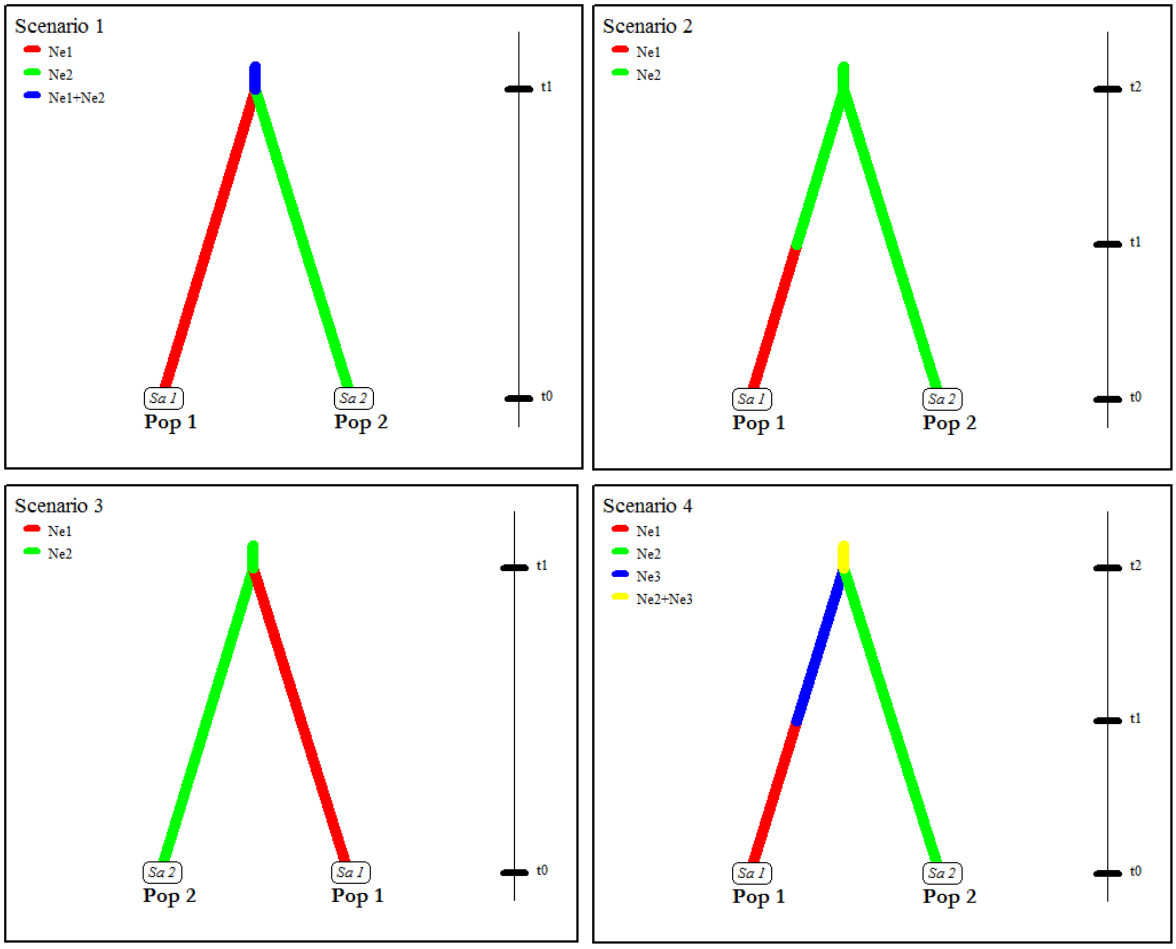


Figure 2.3. Scenarios coded to simulate datasets in DIYABC (Cornuet *et al.*, 2008). Scenario 1 represents simple divergence; Scenario 2 represents divergence followed by a change in the size of one population while the other remains unchanged; Scenario3 represents divergence with simultaneous change in population size for one population with the second unchanged; Scenario 4 represents a variation of scenario 1 with a further reduction in Ne after divergence (see text for more details). Time is not to scale.

representing (Figure 2.3, page 41): 1- simple divergence with the ancestral population effective size (Ne) being the sum of the daughter populations Ne; 2- divergence at time t2 in the past, with one of the populations experiencing a change in Ne later, at t1 in the past; 3- divergence occurring with a simultaneous change in Ne in one of the populations; 4- divergence occurring with a simultaneous change in Ne in one of the populations which later experiences another change in Ne, representing a scenario where the bottleneck was preceded by an earlier divergence. Two sets of simulations were made with 600,000 datasets simulated for each scenario. In one simulation uniform priors were used for all summary statistics, while in the other, Ne and different timings were constrained according to Table 2.4 (this page). The fit of each simulation to the observed data was assessed through a PCA, while the subsequent assessment of which of the scenarios better fitted the data was done using the logistic regression method. Estimates for different Ne and timings was done by averaging such values in the 6,000 closest simulated datasets for the best fitting scenario. All the procedures were implemented in DIYABC software (Cornuet *et al.*, 2008).

Table 2.4. DIYABC (Cornuet *et al.*, 2008) parameter prior distribution for the simulation using constrained priors (see text for description)

Parameter	Distribution	Minimum	Maximum	Additional condition
Ne1	Uniform	10	100	< Ne2
Ne2	Uniform	10	10,000	> Ne1
Ne3	Uniform	10	10,000	—
t0	Uniform	1	10	< t1
t1	Uniform	1	100	> t0; < t2
t2	Uniform	10	10,000	> t1

2.3. RESULTS

2.3.1. Summary statistics and population structure

From the allele number vs. sample size plot, it is evident that there is a tendency for populations with a larger sample size to have higher number of alleles (Figure 2.4, page 43). However, none of these populations showed any significant patterns that appeared to be correlated with sample size (see results). Allele range was increased relative to previous studies in all 15 microsatellites considered for the population structure analysis (Table 2.5, page 43).

G-statistic test of population differentiation showed a significant result, meaning it detected the presence of population structure among populations (Figure 2.5, page 44).

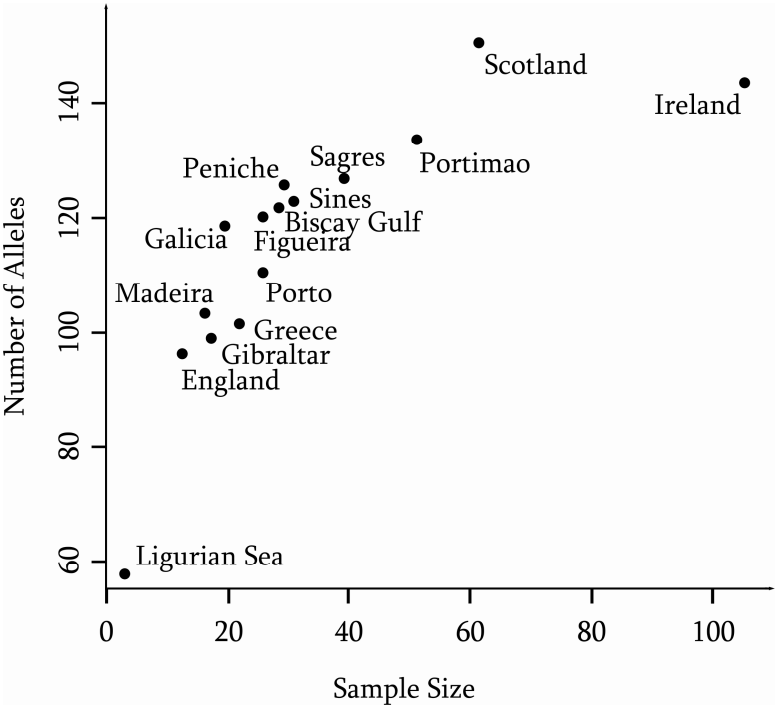


Figure 2.4. Plot of sample size vs. number of alleles.

Table 2.5. Allele range obtained in this study for the 15 microsatellites used in the population genetic analysis.

	Locus Name	Fluorescent Dye	Allele Range
Set A	DO8	FAM	83-123
	KWM2a	FAM	134-170
	KWM2b	HEX	162-180
	KWM12a	NED	154-184
	KWM1b	HEX	181-193
Set B	TtruAAT44	HEX	82-109
	TexVet9	FAM	122-126
	Dde70	HEX	119-165
	Dde84	FAM	143-167
	Dde65	FAM	179-211
	Dde69	HEX	194-226
	Dde09	FAM	218-250
	Dde72	HEX	229-281
	Dde66	FAM	334-374
	Dde59	HEX	310-432

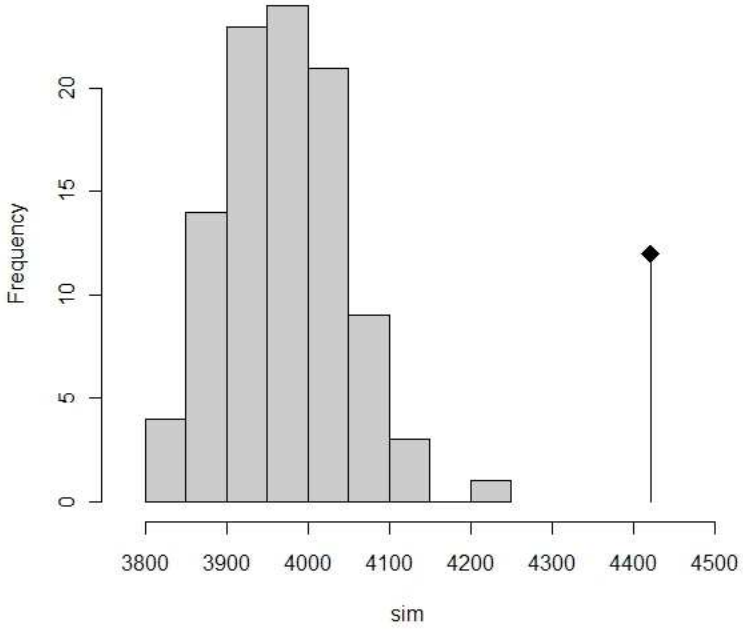


Figure 2.5. G-statistic test plot. Diamond indicates where the data fits within the distribution relative to simulations. Plot drawn using ADEGENET (Jombart, 2008).

Pairwise Fst values between sampling locations are generally low, with the highest values found in comparisons between Greece and other locations (0.032-0.064). Also, only comparisons involving Greece have significant Fst values with the exception of the Ligurian sea (Table 2.6, page 46), which is probably an artefact of low sample size for that region (N=4).

The PCA revealed that the first 2 components only explained 7 % of the variation. Only one group is apparent, although Greek individuals appear to occupy a more peripheral position in the plot along the first component (Figure 2.6, page 47). Ligurian Sea show a similar pattern along the second component, but this is probably due to the low sample size (N=4).

The correspondence analysis showed that 24 % of the variability is explained by the first component, and clearly separates Greece from all the other populations. Interestingly, components 2 and 3 (which explain a further 20 % of the variation) separate Madeira and Scotland respectively, both located at the edge of the geographical distribution of the analysed samples (Figure 2.7, page 47). This result is consistent with the one obtained with the FCA in GENETIX (Figure 2.8, 2.9 & 2.10, page 48).

2.3.2. Individual-based population structure

Using the clustering of individuals algorithm in BAPS (Corander *et al.,* 2008), the most likely number of K was 9 (Probability of K9=0.99). However, from the ancestry plot (Figure 2.11, this page) we can see that the main division is between Greek individuals and all other individuals. It should be noted that samples from Greece are from 2 different geographic locations, 19 from Kalamos in the Ionian Sea, and 3 from the Korinthiakos Gulf, a landlocked bay connected to the Ionian Sea. Notably, the 3 samples collected in the Korinthiakos Gulf cluster in its own group, and 2 other Greek samples cluster outside Greece.

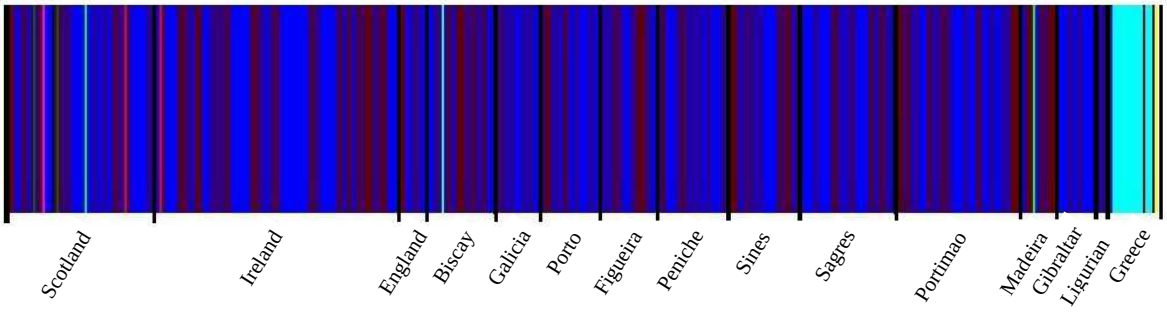


Figure 2.11. BAPS (Corander *et al.,* 2008) ancestry plot for K=9 obtained using the clustering of individuals algorithm.

Table 2.6. Pairwise Fst comparisons between European locations. Fst values are above the diagonal. Significant P-values below the diagonal.

	Portimão	Sagres	Sines	Peniche	Figueira	Porto	Greece	Ligurian	Gibraltar	Madeira	Galicia	England	Scotland	Ireland	Biscay
Portimão	0	0.000438	-0.00017	0.001844	0.00055	0.003719	0.040706	0.007867	0.00866	0.005399	0.00869	0.00079	-0.00149	0.002796	0.002655
Sagres	n.s.	0	0.000421	0.00243	-0.00165	0.00555	0.039399	0.016766	0.010623	0.008331	0.006305	-0.00073	0.001972	0.0009	0.001576
Sines	n.s.	n.s.	0	-0.00327	0.0016	-0.00238	0.039557	0.014336	0.002172	0.001015	-0.00059	-0.00342	-0.00087	-0.00123	-0.00436
Peniche	n.s.	n.s.	n.s.	0	0.001647	0.00377	0.032045	0.007976	0.00087	0.004054	0.002994	-0.0003	0.000207	0.001419	-0.00094
Figueira	n.s.	n.s.	n.s.	n.s.	0	0.007906	0.041021	0.016451	0.003368	0.012049	0.009319	0.000972	0.002289	0.003176	0.002364
Porto	n.s.	n.s.	n.s.	n.s.	n.s.	0	0.04805	0.024174	0.008044	0.002582	0.005898	0.006299	0.002107	0.002894	-0.0011
Greece	0.0105	0.0105	0.0105	0.0105	0.0105	0.0105	0	0.064446	0.035589	0.046992	0.039503	0.047838	0.039886	0.043584	0.044703
Ligurian	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0	0.016073	0.013595	0.02783	-0.00964	0.015829	0.018811	0.019434
Gibraltar	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.0105	n.s.	0	0.018849	0.006304	0.01767	0.010823	0.010162	0.011326
Madeira	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.0105	n.s.	n.s.	0	0.010186	0.003181	0.003524	0.006353	-0.00212
Galicia	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.0105	n.s.	n.s.	n.s.	0	-0.00174	0.004491	0.001797	0.000049
England	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.0105	n.s.	n.s.	n.s.	n.s.	0	0.002509	-0.00182	-0.00441
Scotland	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.0105	n.s.	n.s.	n.s.	n.s.	n.s.	0	0.000203	0.000734
Ireland	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.0105	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0	-0.00289
Biscay	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.0105	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0

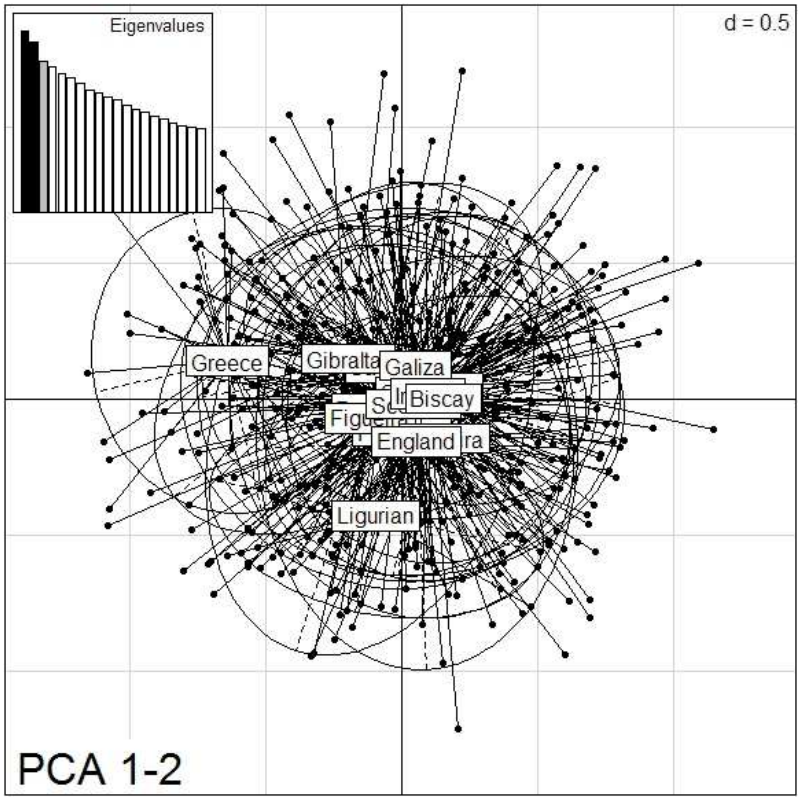


Figure 2.6. Individual based PCA plot comparing all European samples. Components 1 and 2 are represented. Eigenvalues bar plot on the top left. Plot drawn using ADEGENET (Jombart, 2008).

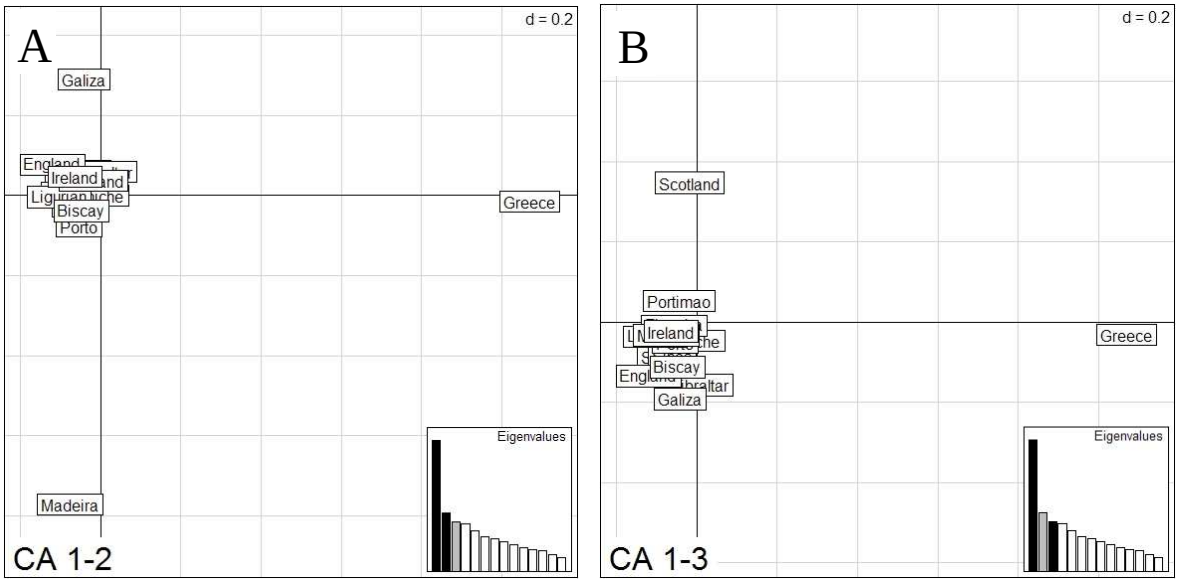


Figure 2.7. Population based correspondence analysis plot. Eigenvalues bar plot on the lower right of each plot. A- Components 1 vs 2; B- Components 1 vs 3. Plots drawn using ADEGENET (Jombart, 2008).

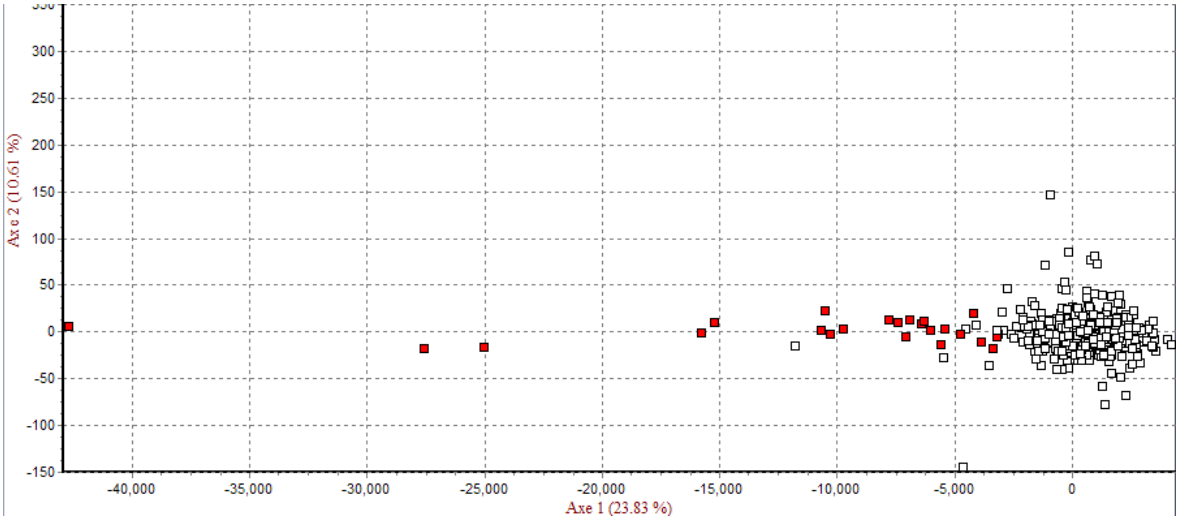


Figure 2.8. Individual based FCA plot. Red represents Greece; white represent all other locations. Plot built using GENETIX (Belkhir *et al.*, 2004).

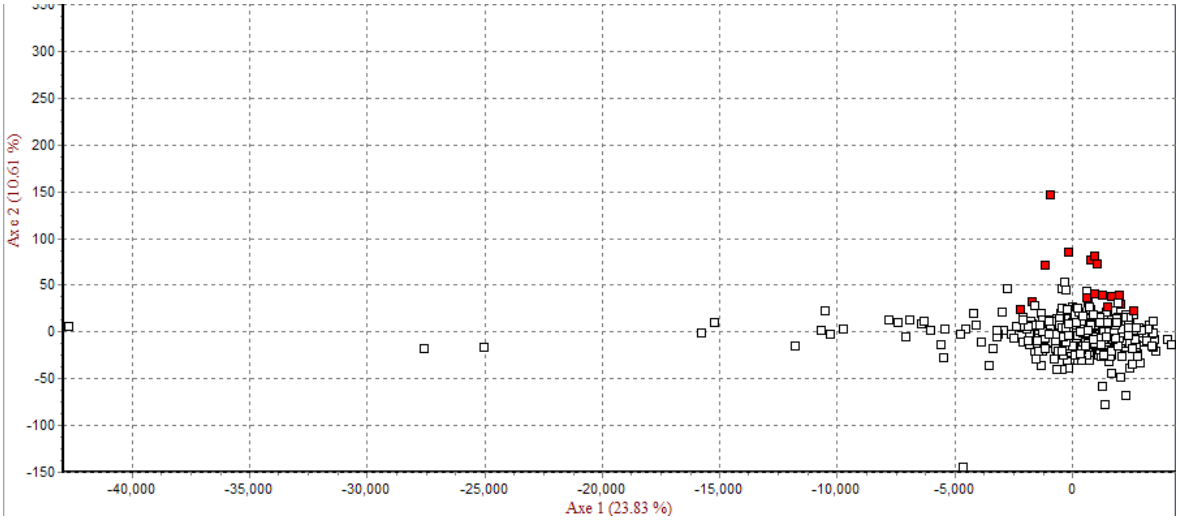


Figure 2.9. Individual based FCA plot. Red represents Madeira; white represent all other locations. Plot built using GENETIX (Belkhir *et al.*, 2004).

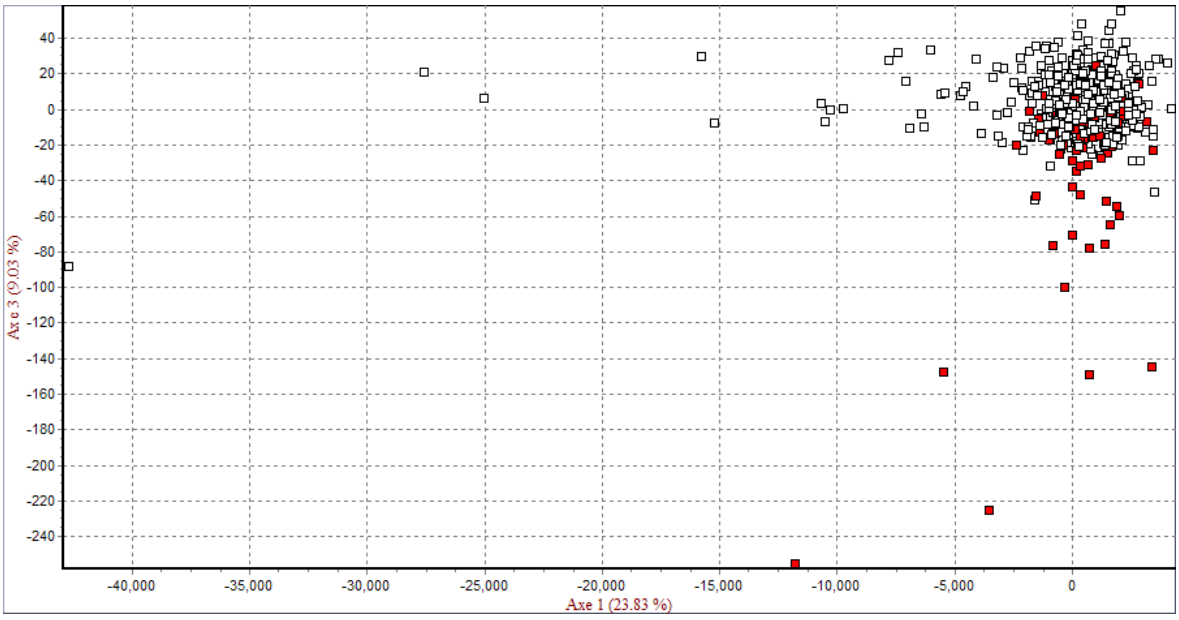


Figure 2.10. FCA Individual based FCA plot. Red represents Scotland; white represent all other locations. Plot built using GENETIX (Belkhir *et al.*, 2004).

The clustering of groups algorithm resulted in a very strong support for K=2 (Probability of K2=1), separating Greece from all other samples (Figure 2.12, this page). This result clearly supports the separation of Greece from the other populations.

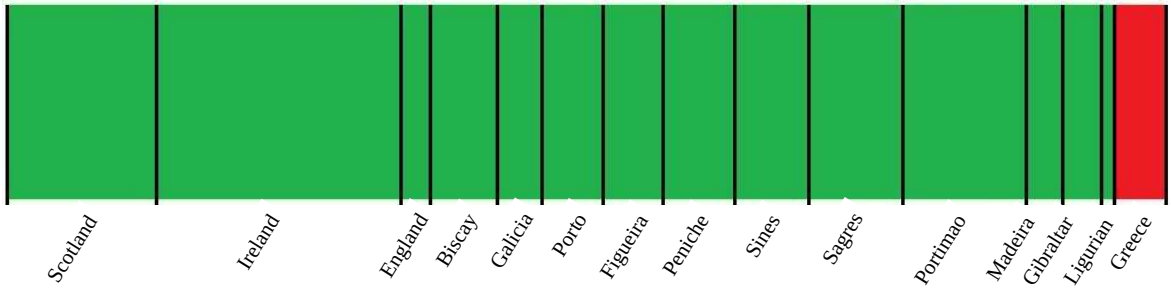


Figure 2.12. BAPS (Corander *et al.*, 2008) ancestry plot for K=2 obtained using the clustering of groups algorithm.

Using the STRUCTURE algorithm (Pritchard *et al.*, 2000), the most likely value for K was 1 (Figure 2.13, this page). All 4 chains achieved similar likelihood values, indicating that enough iterations were performed to reach convergence.

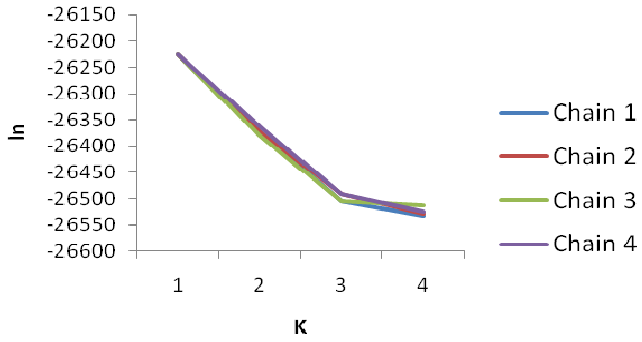


Figure 2.13. Likelihood (Ln) values for each K on all 4 chains implemented in STRUCTURE (Pritchard *et al.*, 2000).

However, Fst values are low, which can undermine the ability of STRUCTURE (Pritchard *et al.*, 2000) to detect subtle population differences, and looking at the plots for higher levels of K provides some consistent patterns. When looking at the bar plot for K=4, it is apparent that Greece separates into its own cluster, with the 3 samples from Korinthiakos Gulf showing distinct ancestry patterns (Figure 2.14, page 50). The triangle plot for K=3 shows a picture somewhat similar to the PCA plot. Ionian Sea Greek individuals do not form an independent cluster, but mostly group at the edge of the main cluster including all other individuals admixed. Korinthiakos samples group with all the others, suggesting that these are more similar to other European samples, and thus masking the differentiation of Greece (Figure 2.15, page 50).

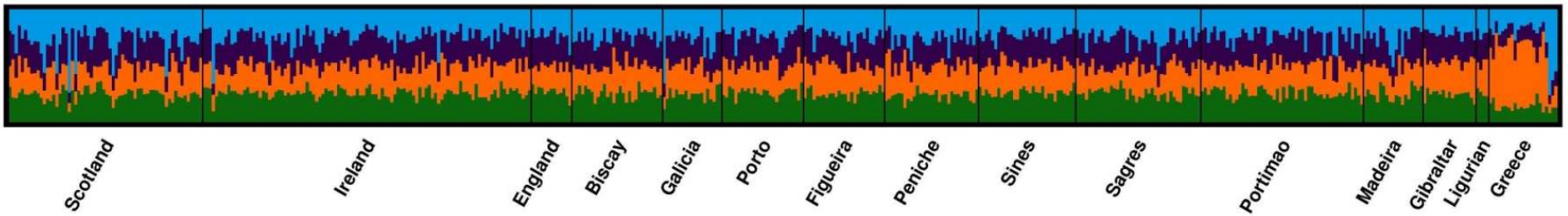


Figure 2.14. STRUCTURE (Pritchard *et al.*, 2000) ancestry plot for K=4. Plot drawn using DISTRUCT (Rosenberg, 2004).

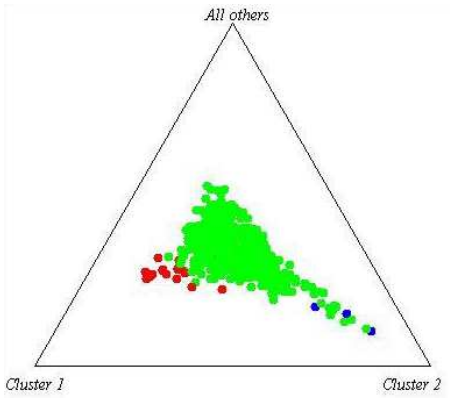


Figure 2.15. STRUCTURE (Pritchard *et al.*, 2000) triangle plot for K=3. Greek individuals from the Ionian Sea represented in Red; samples from the Korinthiakos Gulf in Blue; all others in Green.

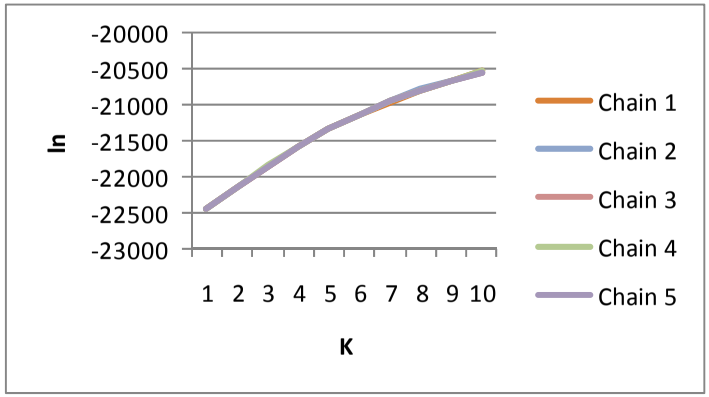


Figure 2.16. Likelihood (Ln) values for each K on all 5 chains implemented in INSTRUCT (Gao *et al.*, 2007).

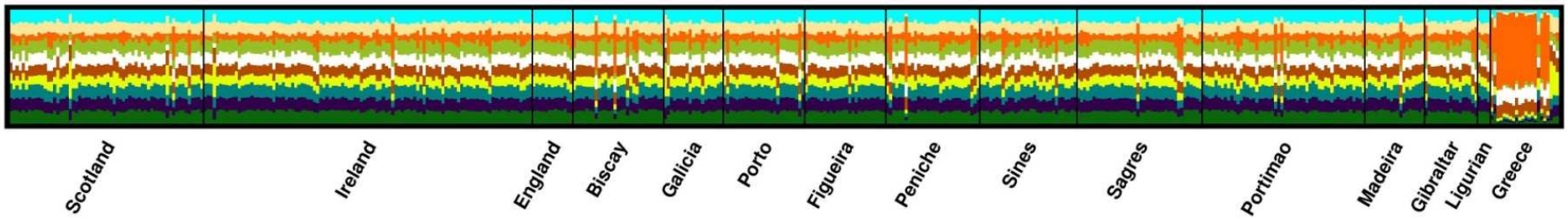


Figure 2.17. INSTRUCT (Gao *et al.*, 2007) ancestry plot for K=10. All the results from K=3 to K=10 showed similar overall results. K=2 only separated Greece for the rest of Europe. Plot drawn using DISTRUCT (Rosenberg, 2004).

Using the INSTRUCT (Gao *et al.*, 2007) algorithm, the most likely value of K determined was 10 (Figure 2.16, page 51). Looking at the population ancestry plot however, it is clear that only Greece separates from the rest of the samples that are strongly admixed, although some European samples exhibit an ancestry pattern more similar to Greece (Figure 2.17, page 51).

It is noteworthy that within Greek samples, one has an admixed ancestry similar to most other European samples, while other appears to have a mixed pattern. These 2 samples correspond to the only 3 samples that did not group as cousins with all the other Greek samples in the KINGROUP analysis (see below). Also, the 3 individuals from the Korinthiakos Gulf have a similar pattern as all other European samples. Notably, in the plot for other values of K, these samples group within their own cluster, also including other European individuals.

2.3.3. Isolation by distance

Neither the Mantel test nor the spatial autocorrelation showed any relationship between genetic and geographic distance (Figure 2.18 & 2.19 respectively, page 53). As such, the observed population differentiation patterns are likely not due to isolation by distance.

2.3.4. Population diversity

Given that a significant population differentiation was only found between Greece and other European locations, genetic diversity statistics were only calculated for Greece, and the other European locations pooled. All diversity estimates calculated show, unsurprisingly, a lower diversity for the Greek population (Table 2.7, this page). However, and in stark contrast with the other statistics, Fis was not only lower in Greece than in Europe, but it was negative as opposed to the positive value of Europe (Table 2.7, this page). Given that Europe shows higher diversity in all the statistics calculated, this result is rather surprising.

Table 2.7. Comparison between Greece and all other European populations for several diversity indices. Na- Number of alleles; Neff- Number of effective alleles; I- Shannon Information Index; Ho- Observed heterozygosity; He- Expected heterozygosity; UHe- Unbiased expected heterozygosity;

Population		Na	Neff	I	Ho	He	UHe	Fis
Greece	Mean	6.867	3.936	1.493	0.703	0.684	0.700	-0.035
	SE	± 0.542	± 0.428	± 0.119	± 0.047	± 0.047	± 0.048	± 0.026
Europe	Mean	12.533	5.492	1.752	0.709	0.734	0.735	0.031
	SE	± 1.369	± 0.663	± 0.164	± 0.059	± 0.061	± 0.061	± 0.012

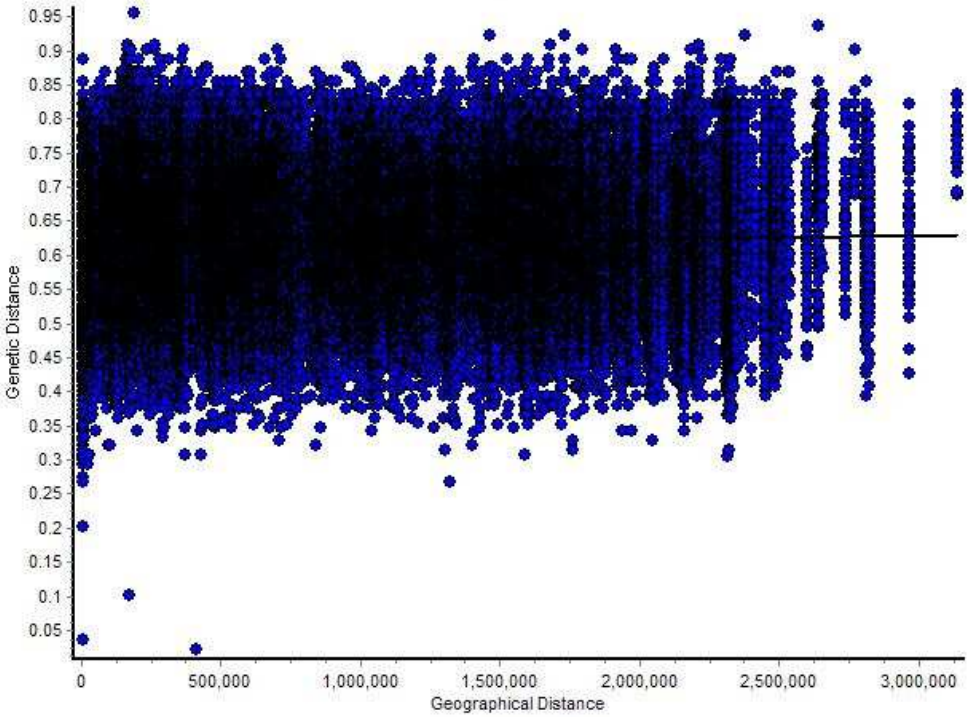


Figure 2.18. Mantel test plot of genetic distances vs. geographic distances. Distance represented in meters. Plot drawn using AiS (Miller, 2005).

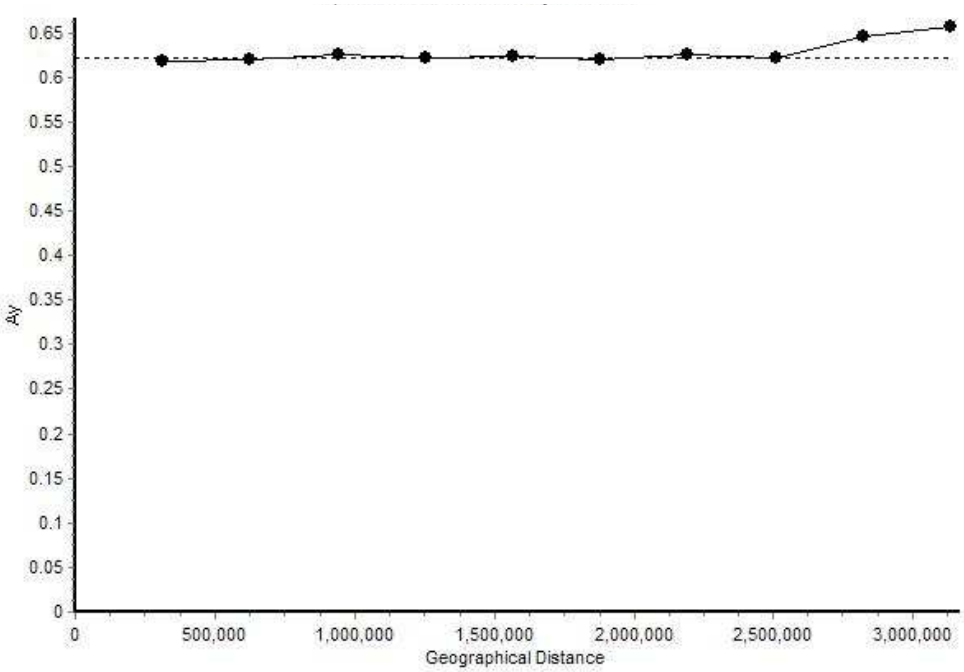


Figure 2.19. Spatial autocorrelation plot. Distance represented in meters. Plot drawn using AiS (Miller, 2005).

2.3.5. Kinship analysis

In the kinship analysis, Greece stands out has having a noticeably higher number of all classes of kinship groups (Figure 2.20, this page). It is noteworthy that only 4 groups of cousins were found in Greece. All these groups included individuals found in other locations around Europe and so were not composed solely of Greek individuals. Notably, individuals sampled in the Korinthiakos Gulf which group in their own cluster in the individual based analyses do not form any kinship group together with other Greek samples. Scotland and Ireland also exhibit a particularly high number of cousin groups, but it is not accompanied by a corresponding higher proportion of other kinship classes.

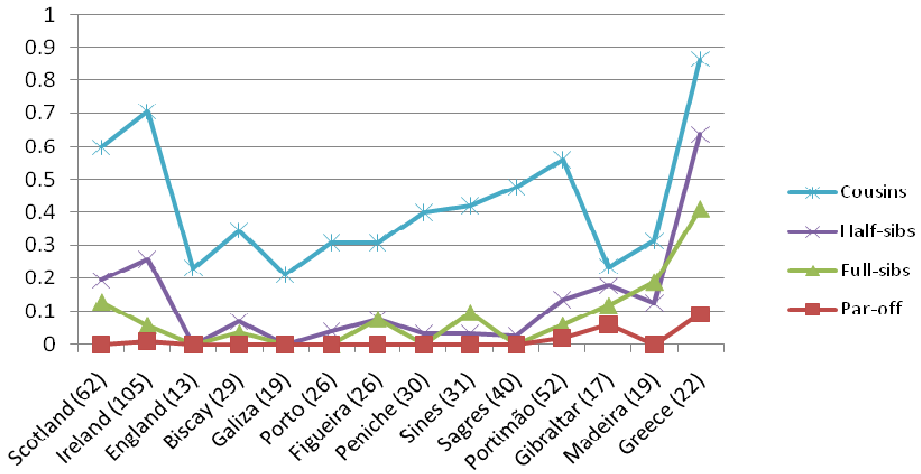


Figure 2.20. Plot representing the proportion of different kinship class for each European location. Number of samples is in Brackets. See Methods for details on the calculations.

The proportion of cousin groups found within each location is correlated with sampling size, explaining the relatively high proportion found in Portimão, Scotland and Ireland. However, the high number of cousin groups in those regions is not accompanied by a higher proportion of other kinship classes, as it is in the Greek sample. Furthermore, the high number of cousin groups in Greece is independent of sample size (Figure 2.21, this page).

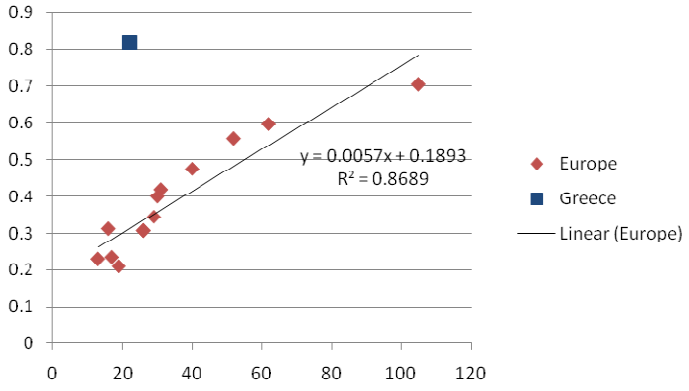


Figure 2.21. Plot of Sampling size vs. Proportion of cousin groups for each European location. Regression was calculated excluding Greece. Greece is included to show its position relative to the calculated regression line.

2.3.6. Estimates of effective population size

In the estimate of effective population size (Ne), Greece is the only location where estimated Ne is lower than the sample size (Table 2.8, this page).

Table 2.8. Estimated effective population size (Ne) for each European location. CI – Confidence Interval.

Population	Sample size	Ne	Lower CI	Higher CI
Scotland	62	126	105.4	154.9
Ireland	105	257.4	207.5	334.4
England	13	47.9	31.6	93.3
Biscay	29	212.4	116.7	963.1
Galicia	19	114.5	68.2	321.8
Porto	26	73.4	54	111.5
Figueira	26	808.9	189.8	Infinity
Peniche	30	233.3	129.5	986.4
Sines	31	180.2	109.1	473.9
Sagres	40	344.5	183.6	2090.2
Portimao	52	367.2	217.5	1071.8
Madeira	16	25.1	20.3	32.1
Gibraltar	17	79.1	48.5	194
Greece	22	17.3	15.2	19.8

The program BOTTLENECK (Cornuet & Luikart, 1996) showed statistical support for a bottleneck in Greece for all mutation models (Table 2.9, page 56), as well as a mode shift in allele frequencies (Figure 2.22, page 56).

In the DIYABC (Cornuet *et al.*, 2008) analysis, the PCA revealed that the simulations with the unconstrained priors (Figure 2.23, page 57) had a worse fit than the constrained priors (Figure 2.24, page 57), and as such the remaining analyses were based on the scenarios from the later simulations. The logistic regression evaluation of the different constrained scenarios revealed that scenario 2 was the one who best fitted the observed data (Figure 2.25, page 58), thus supporting the idea of a bottleneck in the Greek population. The parameter estimate revealed that Ne values for both populations is proportionally consistent with the one obtained by the program BOTTLENECK (Cornuet & Luikart, 1996)(meaning a much lower value for the Greek population than for the European one), and that both the divergence between the populations and the reduction in Ne in the Greek population were very recent (Figure 2.26, page 58).

Table 2.9. Significance test results from BOTTLENECK (Cornuet & Luikart, 1996). Expected Het. Exc. – Number of *loci* with heterozygote excess expected for each mutation model; Observed Het. Exc. – Number of *loci* with heterozygote excess observed in the data for each mutation model; Sign. – p-values of the significance test; Stand. Diff – p-values for the standardized differences test; Wilcoxon H. Def – one tail p-value for heterozygotes deficiency; Wilcoxon H. Exc. – one tail p-value for heterozygotes excess; Wilcoxon Two tail – two tail p-value for both heterozygotes deficiency and excess

Mutation Model	Expected Het. Exc.	Observed Het. Exc.	Sign.	Stand. Diff.	Wilcoxon H. Def.	Wilcoxon H. Exc.	Wilcoxon Two-tail
I.A.M.	8.05	15	0.00008	0.00000	1.0	0.00002	0.00003
T.P.M.	8.51	14	0.00246	0.00019	0.99998	0.00003	0.00006
S.M.M.	8.57	13	0.01569	0.00534	0.99934	0.00084	0.00168

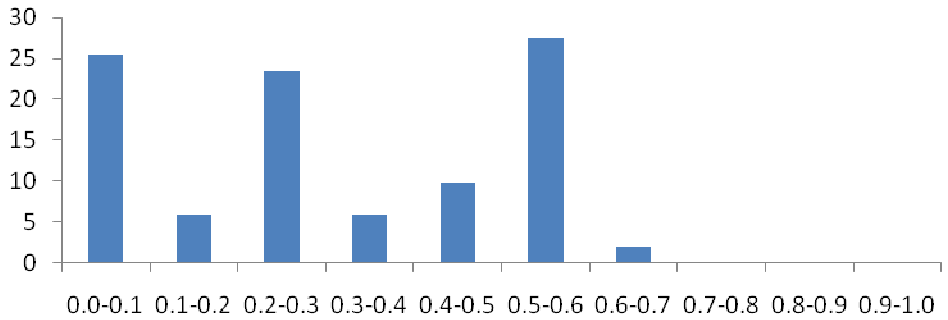


Figure 2.22. Allele frequency distribution for the Greek population as determined by BOTTLENECK (Cornuet & Luikart, 1996). Frequency classes represented in the x-axis, while percentage of alleles in each class represented in the y-axis. Stable populations are expected to exhibit an L-shaped distribution (Luikart & Cornuet, 1998).

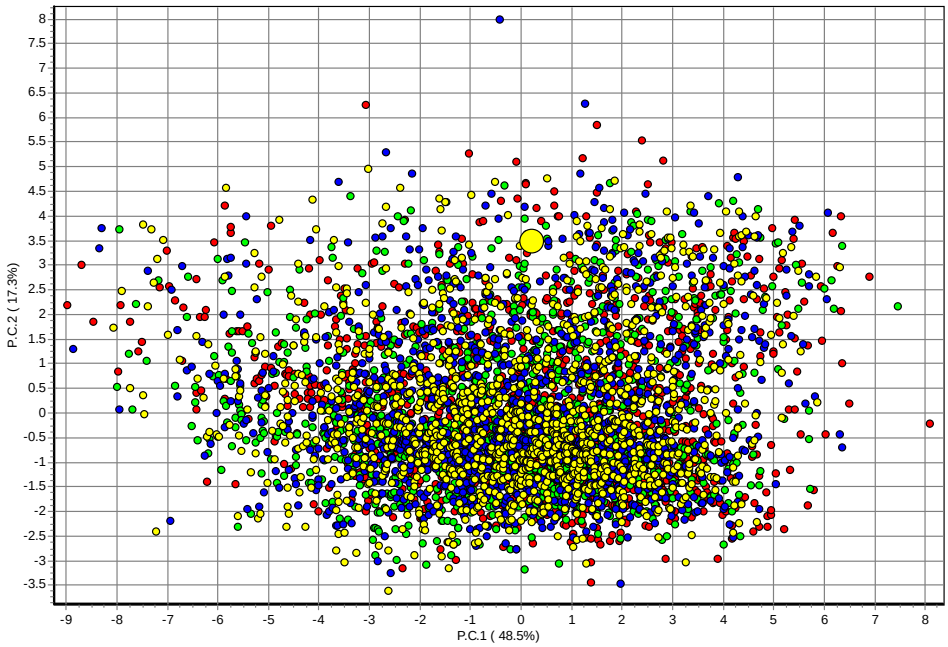


Figure 2.23. PCA plot displaying the fit between scenarios simulated with uniform unconstrained priors and the observed data. Large yellow dot represents the observed data, while small dots represent the several simulated datasets. Plot made using DIYABC (Cornuet *et al.*, 2008). Simplified plot available in Appendix 2.1, page 141.

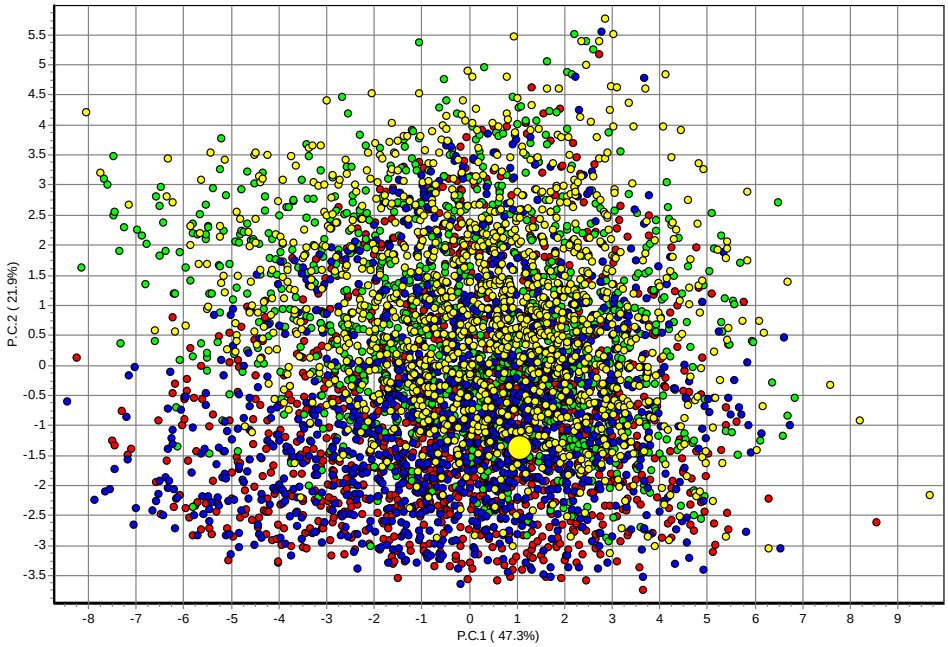


Figure 2.24. PCA plot displaying the fit between scenarios simulated with constrained priors and the observed data. Large yellow dot represents the observed data, while small dots represent the several simulated datasets. Note that the observed data fits the simulated datasets much better than the unconstrained simulations in Figure 2.23. Plot made using DIYABC (Cornuet *et al.*, 2008). Simplified plot available in Appendix 2.1, page 141.

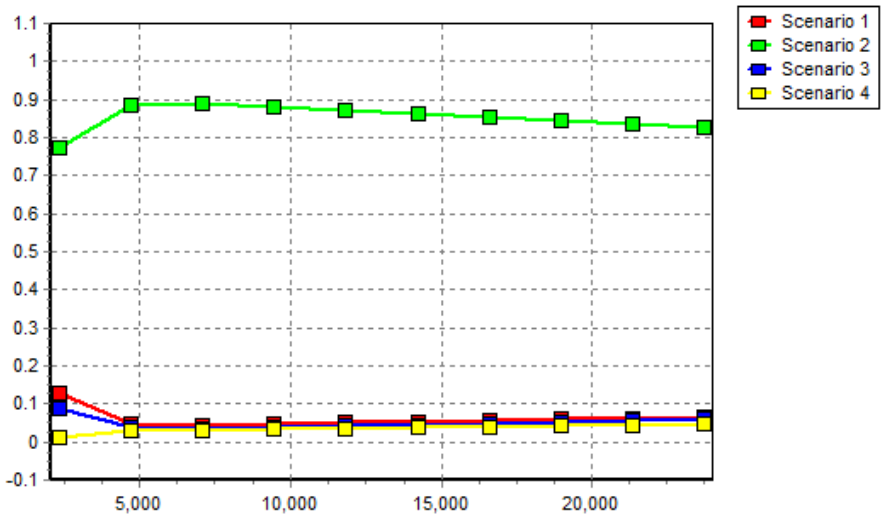


Figure 2.25. Logistic regression plot to test the fitness of the observed data and simulated scenarios.Plot drawn in DIYABC (Cornuet *et al.*, 2008).

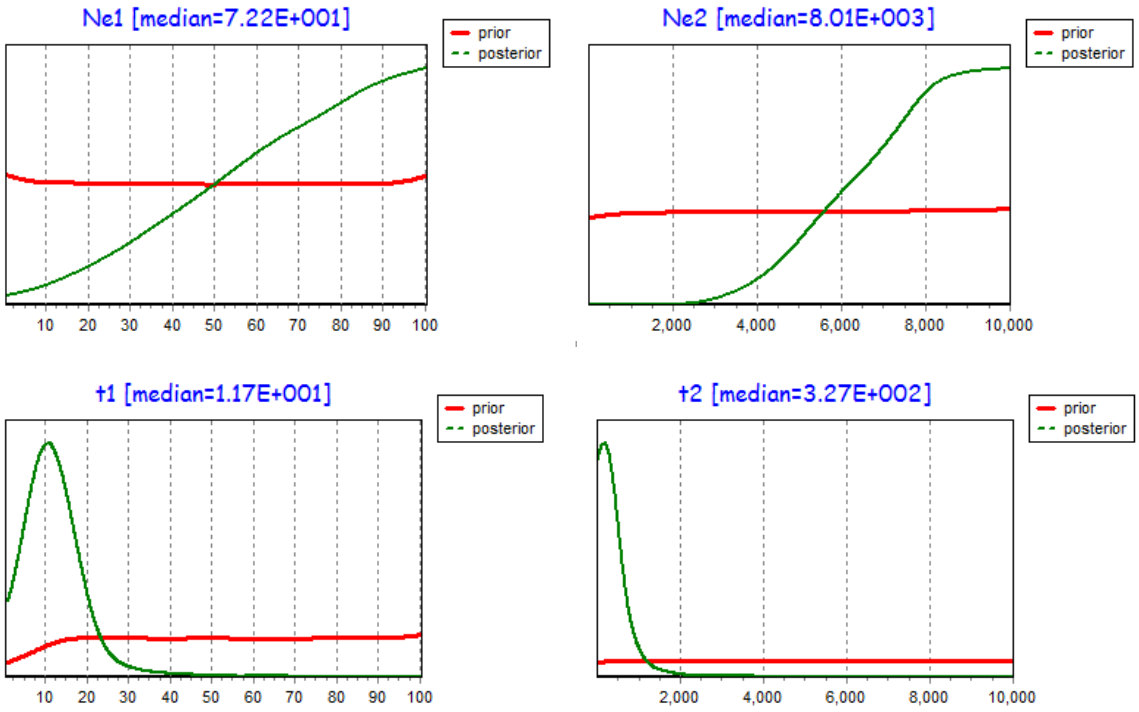


Figure 2.26. DIYABC (Cornuet *et al.*, 2008) plot of parameter estimate using the 6000 simulated datasets under scenario 2 that were closer to the observed data. The x-axis range reflects the prior distribution.

2.4. DISCUSSION

The results clearly show a lack of population genetic structure along the European coastline for the common dolphin. Only Greece seems to differentiate, but the differentiation is nevertheless weak. Fst comparisons between Greece and other locations are generally significant and within the ranges found for other dolphin species in Europe (0.032-0.064 in this study; 0.045-0.152 for bottlenose in similar areas (Natoli *et al.*, 2005); 0.0047-0.0632 in striped dolphin (Gaspari *et al.*, 2007)). The test for population structure using G-statistics confirmed this result by showing significant signals of population structure. This, however, only shows that allele frequencies are different in Greece. The individual based clustering algorithm implemented in STRUCTURE (Pritchard *et al.*, 2000) did not find statistical support for that differentiation, while BAPS (Corander *et al.*, 2006) not only differentiated Greece, but further supported a division between the Ionian Sea and the Korinthiakos Gulf. The pattern obtained for the rest of Europe made little biological sense, given it consists of relatively homogeneous admixture between clusters that no other biological study in Europe to date gave any indication of existing. This is expected to happen with such algorithms when Fst values are lower than 0.1 (Corander, 2006; Latch *et al.*, 2006) as is the case of the current dataset. INSTRUCT (Gao *et al.*, 2007), which takes into account population structure due to inbreeding, gave strong support for the separation of Greece, although some European individuals showed an ancestry pattern similar to Greece. The Korinthiakos Gulf samples, however, grouped with the rest of Europe. In the PCA, Greek individuals do not fully separate from the rest of Europe, but occupy a peripheral position in the single cloud of points. CA and FCA confirmed this, also placing Madeira and Scotland (locations at the limit of the analysed geographical distribution) at the edge of the plot, but only Greece separated consistently regardless of the factors compared.

The lack of differentiation across Europe except between the Eastern and Western Mediterranean Sea confirms the patterns observed previously in the common dolphin (Natoli *et al.*, 2006; Natoli *et al.*, 2008; Mirimin *et al.,* 2009). The detailed sampling scheme used in this study for the Iberian coast, showed that the previous suggestion of fine-scale population structure in that region (Amaral *et al.*, 2007) cannot be supported by the data presented here. Other cetacean species have been found to exhibit barriers to gene flow in the same geographical area as observed here for the common dolphin (between Greece and the rest of Europe) (Natoli *et al.*, 2004; Natoli *et al.*, 2005; Gaspari *et al.,* 2007). However, such species usually exhibit further population structure elsewhere in the European coast, namely between

the Mediterranean and the Atlantic, and between the Atlantic and the UK. In fact, the transition from the Mediterranean Sea to the Atlantic Ocean (roughly represented by the Strait of Gibraltar) appears to be a strong barrier to gene flow in marine organisms. Several cetacean species analysed exhibit population structure between the Mediterranean Sea and the Atlantic Ocean (Bérubé *et al.,* 1998; Garcia-Martinez *et al.*, 1999; Natoli *et al.*, 2004; Valsecchi *et al.*, 2004; Dalebout *et al.*, 2005; Natoli *et al.*, 2005; Gaspari *et al.*, 2007; Engelhaupt *et al.*, 2009), as do other marine organisms such as several species of fish (Borsa *et al.*, 1997; Chikhi *et al.*, 1997; Roldan *et al.*, 1998; Naciri *et al.*, 1999; Carreras-Carbonell *et al.*, 2006; Charrier *et al.*, 2006; Abaunza *et al.,* 2008), cuttlefish (Perez-Losada, 2002) and mussels (Quesada *et al.*, 1995). Surprisingly, common dolphin appears to exhibit no population structure across this barrier, while apparently having structure within the Mediterranean Sea.

Kinship analysis showed that Greek individuals are all much more strongly related to each other than individual samples in other locations. Interestingly, individuals from the Korinthiakos Gulf are not related to the ones form the Ionian Sea, even though these sea basins are connected. Consistently, statistics of genetic diversity all indicate that Greece is less diverse when compared to the rest of Europe, while Fis in Greece is much lower. This suggests that the Greek population has undergone some sort of population reduction, either due to a founder event or a bottleneck. However, several lines of evidence suggest that the Greek population was historically connected with the other European populations, but has recently undergone a severe bottleneck. In populations with large sizes, rare alleles will be common but contribute little to heterozygosity. When such populations undergo a fast and severe reduction in size, allele frequencies will change drastically, with the rarest alleles usually disappearing first. As such, heterozygosity will remain high proportionally to the reduced allelic diversity (Maruyama & Fuerst, 1985; England *et al.,* 2003). This was strongly supported by the BOTTLENECK (Cornuet & Luikart, 1996) analysis which detected a significant excess of heterozygotes for all mutation models as well as a shift in the allelic frequency distributions. Also, when comparing allele frequencies across *loci* between Greece and other European populations, it is apparent that many of the rare alleles found in Europe are not found in Greece (Appendix 2.2, page 142). When comparing diversity statistics between Greece and Europe, it is also clear that observed heterozygosity is much less reduced in Greece, when compared to number of alleles, allele-richness and expected heterozygosity. Finally, the estimated effective population size (Ne) for Greece is lower than sample size. Because a positive relationship between samples size and allele number was found in present dataset, the

observation that everywhere else in Europe Ne is much higher than sampling size probably reflects that sampling as not been sufficiently representative to in all these locations to accurately calculate Ne based on a point calculation method used in this study. However, with a sampling number of 22, the lower Ne obtained for Greece probably reflects an accurate calculation, and it likely reflect a very severe reduction suffered by this population . In such a scenario, genetic variation would be reduced, but after a few generations of breeding, the population would be composed of related individuals with highly heterozygous genotypes when compared to the allelic variation, leading to lower diversity but negative Fis values. However, mating among relatives is expected to increase Fis rapidly, meaning that the negative value of Fis for the Greek population would only last a short period of time, thus suggesting a very recent bottleneck. This scenario was confirmed by the Approximate Bayesian Computations, where datasets simulated under the assumption of a recent strong reduction in one of the diverging population fitted the data better than scenarios where no such assumptions were made. The scenario that best fitted the data suggests, however, that divergence was already occurring before the bottleneck occurred. This can reflect a real demographic pattern, but the recent dates for both the divergence (around 300 years ago) and the bottleneck (around 11 years ago) are also compatible with a continuous population decline over the past hundred years rather than an instantaneous bottleneck causing an immediate divergence between the two populations (as simulated in alternative unsupported scenarios).

The genetic evidence suggesting a bottleneck for the Greek population is consistent with independent demographic data obtained from the Mediterranean common dolphin, where a population reduction has indeed been reported. Common dolphin is thought to have been abundant throughout the Mediterranean Sea, although such statements are usually based on indirect historical records (e.g., strandings and museum collections) (Forcada & Hammond, 1998; Bearzi *et al.,* 2003; Bearzi *et al.*, 2004). However, more recent and direct evidence suggests a continuing decline in the Mediterranean population to the present day. Strandings in the Mediterranean coast of France have been declining since the 1970's (Bearzi *et al.*, 2003), while transect observations recorded an extremely low incident in Italian waters where the species was once abundant (Notarbartolo-di-Sciara *et al.*, 1993). Very few animals are also reported from the Adriatic Sea (Bearzi *et al.,* 2003; Bearzi *et al.*, 2004). More notably though, and more relevant in the context of this study, the decline of common dolphins in the Ionian Sea around the island of Kalamos has been directly observed. During extensive population surveys, the mean encounter rate has dropped dramatically to the point that it became an

extremely rare sighting compared to only a few years before (Bearzi *et al.,* 2005; Bearzi *et al.*, 2006; Bearzi *et al.*, 2008). This is precisely the population from which the majority of the Greek samples used in this study were obtained. Our results show that the perceived decline in common dolphin numbers is reflected in the genetic data. It is not clear whether the common dolphin populations experienced a population crash or just migrated to other regions in the Mediterranean Sea (little is known of cetacean occurrence along the African coast) but this study seems to support a significant population crash. If individuals had only migrated elsewhere, then the genetic patterns observed in Greece would not be so evident due to gene flow with a larger population. This study also suggests that the bottleneck was very severe and probably occurred over a very small number of generations (England *et al.*, 2003), but further tests are needed to confirm this hypothesis.

Common dolphin exhibits a notorious lack of detectable structure in European waters when compared to other cetaceans (Garcia-Martinez *et al.*, 1999; Natoli *et al.*, 2004; Valsecchi *et al.*, 2004; Natoli *et al.*, 2005; Fontaine *et al.*, 2007; Gaspari *et al.*, 2007; Banguera-Hinestroza *et al.,* 2010), which is probably due to its more generalist diet (Young & Cockcroft, 1994; Ohizumi *et al.*, 1998; Silva, 1999; Meynier, 2004; De Pierrepont *et al.,* 2005; Pusineri *et al.*, 2007) in combination with an extremely fluid social structure and promiscuous mating (Murphy *et al.,* 2005; Westgate & Read, 2007; Viricel *et al.*, 2008). Other cetaceans that also have high dispersal abilities such as the sperm whale (*Physeter macrocephalus*) or the bottlenose dolphin (*Tursiops truncatus*), will still exhibit structure in Europe, probably due to their more selective mating systems and more cohesive social structure (Natoli *et al.*, 2005; Engelhaupt *et al.,* 2009). Although the genetic differentiation found between Greece and the rest of Europe is weak, from the ecological point of view, it is clear that the Greek population is now isolated from the rest of Europe, and given its extreme decline, it is rightly considered a separate management unit (Bearzi, 2003). It has been proposed before that founder events caused population structure in cetaceans (Hoelzel *et al.*, 1998; Gaspari *et al.*, 2007; Banguera-Hinestroza *et al.,* 2010). In the common dolphin, Mirimin and collaborators (2009) suggested that the decline might have had a similar role in the differentiation of Greek individuals (Natoli *et al.*, 2008), which is confirmed by this study. As such, population fluctuations might be a relevant mechanism promoting differentiation in cetaceans (Templeton, 1980; Storz, 1999), including other regions where differentiation was found in the common dolphin (Rosel *et al.*, 1994; Chivers *et al.*, 2005; Bilgmann *et al.,* 2008). Notably, in one case the differentiated population is known to be under the impact of excessive bycatch (Bilgmann *et al.*, 2008). In

the bottlenose dolphin, a species that usually exhibits more pronounced population structure than the common dolphin, it has also been suggested that past population fluctuations have caused local extinctions (Nichols *et al.*, 2007). It remains unclear if selection would have had an important role in this process. It has been suggested that the differentiation found in common dolphins between Greece and the rest of Europe might relate to environmental differences (Natoli *et al.*, 2008), and several other species exhibit patterns consistent with this interpretation (Natoli *et al.*, 2004; Natoli *et al.*, 2005; Perez-Losada *et al.*, 2007; Abaunza *et al.,* 2008). The results from this study further suggest that population fluctuations might have contributed to the differentiation of Greek populations in addition to environmental differences.

Chapter 3 – Analysis of Functional Diversity in the European Common Dolphin (*Delphinus delphis*), with Detection of Selection Signals on Ecologically Relevant Candidate Markers

3.1. INTRODUCTION

When assessing the role of ecological barriers in promoting differentiation in wild cetaceans, most studies use markers that exhibit a relatively high substitution rate (such as mitochondrial DNA or microsatellites) to establish population differentiation, correlating the patterns found with breaks in environmental characteristics (Torres *et al.*, 2003; Natoli *et al.*, 2004; Natoli *et al.*, 2006; Bilgmann *et al.,* 2007b; Fontaine *et al.*, 2007; Natoli *et al.*, 2008; Fontaine *et al.*, 2010). However, such markers are essentially neutral (Avise *et al.,* 1987; Jarne & Lagoda, 1996), and are therefore unlikely to reflect local adaptation unless divergent selection is strong relative to levels of migration and recombination (Kelly, 2006; Thibert-Plante & Hendry, 2009; Thibert-Plante & Hendry, 2010). Even then, when combined with scenarios such as complex geological history or historically small effective population sizes, distinguishing between selection and genetic drift becomes virtually impossible (Coyne & Orr, 2004; Thibert-Plante & Hendry, 2010). It is nevertheless important to understand the patterns of genetic differentiation that may result from the process of natural selection, both for the understanding of evolutionary processes and to promote effective conservation strategies. For that purpose, focus should ideally be on functional genes coding for phenotypic attributes relevant for the environmental differences experienced along a species geographical range (Lewontin, 1974; van Tienderen *et al.*, 2002).

Such an approach is, however, far from straightforward, mainly because identifying the precise relationship between genotype and phenotype has proven an extremely elusive and technically challenging objective (Lander & Schork, 1994; Glazier *et al.*, 2002; Andersson & Georges, 2004). First, several phenotypic characteristics are determined by complex interactions between several different genes (epistasis) (Cordell, 2002) each also potentially involved in more than one trait (pleiotropy) (Caspari, 1952). Mammalian coat colour, for example, is determined in such a fashion. Specific coat colours are determined by the type of melanin being produced in melanocytes, following a complex cascade of reactions requiring the interaction of several different proteins (Slominski *et al.*, 2004). Genes affecting coat

colour will also often influence other unrelated traits, such as obesity (Voisey & Van Daal, 2002). Additionally, phenotypic differences are often controlled by differences in expression of the protein rather than its nucleotide sequence (King & Wilson, 1975), as is the case of differences between the brains of humans and chimpanzees (Caceres *et al.*, 2003) (although some studies report that differences in expression are themselves controlled by single nucleotide polymorphism (Kudaravalli *et al.*, 2009)). Several genes also exhibit multiple exon/ intron splicing varieties, making it impossible to know the exact composition of a protein being expressed from the genomic DNA sequence (Bultman *et al.,* 1994; Mironov *et al.*, 1999; Kampa *et al.*, 2004). Finally, many phenotypic traits are influenced not only by genetics, but also by the surrounding environment. This means that visible differences in phenotype might not be controlled by genetic differences (epigenetics) (Choi & Kim, 2007; Assfalg *et al.,* 2008).

3.1.1. Strategies to investigate ecologically relevant diversity in functional genes

Because phenotypic traits will often be controlled by several different genes, investigating the genetic basis of phenotypic traits is ideally done through QTL mapping and association studies (Lewontin, 1974; Darvasi & Pisanté-Shalom, 2002; Glazier *et al.*, 2002; Mackay, 2002). Once a particular quantitative phenotypic trait has been identified as relevant, it is possible through pedigree analysis to identify regions in the genome which seem to be segregating with the quantitative trait (hence receiving the name of Quantitative Trait *Loci* - QTL). Then association studies between mutations in the QTL and specific phenotypic traits can be made (Darvasi & Pisanté-Shalom, 2002; Glazier *et al.*, 2002; Mackay, 2002; Andersson & Georges, 2004). Such studies can then be followed by functional association and sequence association analyses, in order to identify the extent to which each particular gene (called candidate gene) contributes to the trait of interest (e.g. Andersson & Georges, 2004; Peretz *et al.*, 2007; Peiro *et al.*, 2008; Fontanesi *et al.*, 2009). However, such an approach is very difficult to achieve in wild non-model organisms, and to date has only been achieved fully in laboratory model organisms e.g. yeast (Steinmetz *et al.*, 2002). QTL's identified through pedigree studies usually include thousands of genes which are impractical to analyse in a population study, and the pedigree information required for such studies is often not available or very difficult to gather (Andersson & Georges, 2004). Studies on wild animals have mainly focused on easily accessible species that are closely related to well studied domestic animals, but even those face limitation and have had mixed results. Notably, QTL associations studies are rarely replicated in different populations of the same species, and the ones who have been replicated found no

consistent associations in all populations analysed (Vasemagi & Primmer, 2005; Slate *et al.,* 2010).

Given such limitations, most studies of functional genes in wild populations are mainly restricted to the last step of the process; the sequence analysis of candidate genes. In this case, information on well described genes is used to make an informed speculation on its potential relevance for the adaptation to a specific environment in the wild (Dalziel *et al.*, 2009). Such functional genetic markers can then be used to look for patterns of differentiation or for molecular signatures of selection and relate the findings with particular environments, usually above the species level (e.g. Ward *et al.*, 1997; Juarez *et al.,* 2008; Maheshwari *et al.*, 2008). Recent developments in genomic sequencing have greatly increased the available information on specific genes, making such an approach more tractable and focused (Dalziel *et al.,* 2009; Slate *et al.*, 2010). It has also allowed a more bottom-up approach to be used, where selection detection algorithms are applied to data from genome scans (Nielsen, 2001; Akey *et al.,* 2002; Sabeti *et al.*, 2002; Beaumont & Balding, 2004; Storz, 2005; Hans, 2008; Zayed & Whitfield, 2008). Regions were selection is detected can then be compared to annotated genomes of closely related species to identify and characterize the functional genes in which some of these regions are found (Andersson & Georges, 2004; Vasemagi & Primmer, 2005; Dalziel *et al.*, 2009).

3.1.2. Methods to detect selection in the genome

Different methods exist to detect selection in the genome, either in candidate markers or genome scans. When analysing sequence data, several tests exist that rely on allele frequency or DNA divergence data (e.g. Lewontin & Krakauer, 1973; Watterson, 1977; Hudson *et al.*, 1987; Tajima, 1989; Fu & Li, 1993). However, such tests suffer from very low power to detect real cases of selection (Zhai *et al.*, 2009), and positive results can be created by extreme demographic events or cryptic population structure (Kreitman, 2000; Nielsen, 2001; Ford, 2002). The most direct method to assess positive selection based on sequence data looks at the patterns of substitutions within a given sequence. Due to the redundancy in the genetic code, some substitutions will not result in an aminoacid change, thus resulting in no functional changes in the protein and receiving the designation of 'synonymous'. In contrast, substitutions that do cause an aminoacid change are called 'non-synonymous' (Lewin, 2004). In a neutrally evolving *locus*, both types of substitutions are equally likely to become fixed in the population, and as such, the proportion between non-synonymous substitutions and

synonymous substitutions (dN/dS) should not significantly differ from 1. Because there are less non-synonymous sites in the genetic code, dN/dS is scaled to the availability of non-synonymous and synonymous sites. If the gene is under purifying selection, then non-synonymous mutations are very unlikely to become fixed in the population, and the dN/dS ratio should be lower than 1. If there is selective pressure for the gene to diversify (either through balancing selection or directional selection on new mutations), then dN/dS ratio will be higher than 1 (Hughes & Nei, 1988; Goldman & Yang, 1994; Muse & Gaut, 1994). Maximum likelihood algorithms that test different dN/dS distributions along a sequence in a phylogenetic tree, have been shown to be relatively powerful and largely independent from demography or population structure (Yang & Bielawski, 2000; Wong *et al.,* 2004). In addition, such algorithms also identify the exact codons that are under selection, as well as specific branches in a phylogenetic tree (Yang & Nielsen, 2000; Nielsen, 2001; Yang & Nielsen, 2002; Yang *et al.*, 2005). Numerous studies using dN/dS analysis have found evidence of selection in functional genes, either in candidate genes (Ward *et al.*, 1997; Rooney & Zhang, 1999; Hashiguchi *et al.*, 2007; Ali & Meier, 2008; Juarez *et al.*, 2008; Maheshwari *et al.*, 2008; Dayo *et al.*, 2009; Larmuseau *et al.*, 2010) or through genome scans (Nielsen *et al.*, 2005).

3.1.3. Selection in cetacean genomes

In their transition from a terrestrial to an exclusively marine environment, cetaceans have undergone drastic morphological and physiological changes, involving adaptation in physiological functions such as vision, respiration, thermoregulation, osmoregulation, etc. (Thewissen, 1998; Hoelzel, 2002). Such modifications are expected to leave a clear mark in the genome, such as the evidence for strong selection in cetaceans at the homeobox genes, responsible for the development of limbs in vertebrates (Wang *et al.*, 2009). Even at the population level, such signals of adaptation to different environments have been found in cetaceans, namely in the peptide binding region of the DQβ1 *locus* suggesting adaptation to different pathogenic environments (Vassilakos *et al.*, 2009). Other genes, such as haemoglobin and myoglobin (proteins essential for providing oxygen to skeletal muscle) are known to exhibit structural differences that can sometimes be related to regions with different oceanographic characteristics (Wang *et al.*, 1977; Iwanami *et al.,* 2006; Remington *et al.*, 2007). As such, genes related to well characterized physiological functions might also show signals of selection, not only on a phylogenetic scale, but also on a population scale within

cetacean species that have adapted to different habitats, such as deep diving vs. shallow diving populations. Several such potential functions are discussed in the following sections.

3.1.4. Ecologically relevant candidate genes

Osmoregulation

From the many adaptations that cetaceans had to go through in their transition from land to sea, one of the most significant was the survival in a hyperosmotic environment. Vertebrates in general cannot ingest saltwater, as the resulting excess salt would lead to increased urine production and further cellular dehydration (Randall *et al.*, 2002). Marine birds and reptiles do drink seawater, but they excrete the excess salt through specialized glands (Randall *et al.*, 2002). Cetaceans do not have such systems, but physiological studies have reported the active intake of saltwater (Hui, 1981; Kjeld, 2003). It is thus expected that cetaceans exhibit some adaptations to cope with the specific constraint the marine environment poses to osmoregulation.

In several desert mammals, production of a highly concentrated urine is a common strategy to prevent dehydration (Randall *et al.*, 2002). Urea is one of the main solutes of urine, and as such, the ability to concentrate urea in parts of the nephron is thought to be essential in the osmotic reabsorption of water, thus producing concentrated urine (Fenton & Knepper, 2007), a process which is largely controlled by proteins called "urea transporters". Cetaceans have a higher concentration of urea in plasma and urine than cattle, a phylogenetically related terrestrial group (Birukawa *et al.*, 2008). Birukawa and collaborators (2008) hypothesized that the urea transporters in the kidney might play a role in the urine concentrating abilities of cetaceans (Ridgway & Venn-Watson, 2010), given that differences in urine concentration between baleen whales and sperm whales were related with differences in UT-A2 gene sequence (Birukawa *et al.*, 2008). Additionally to urea transporters, aquaporins are transmembrane channels known to actively promote the transfer of water and other solutes across membranes in the kidney nephron. Although AQP1, the best characterized aquaporin, exclusively transports water, other aquaporins transport glycerol and other solutes together with water (Nielsen *et al.*, 2002).

Milk proteins

Milk production is one of the most crucial physiological functions in mammals. Neonates are exclusively dependent on their mother's milk to survive, and differences in milk quality can have a very big influence on offspring development and survival. Studies in humans have

shown that milk composition influences infant growth rate and overall height attained as an adult (Kuschel & Harding, 2000; Kuschel & Harding, 2004). In addition to nutrition, milk also has an important immunological function in neonates (Hylander *et al.*, 1998).

Cetaceans live in a harsh environment where newborns need to gain weight quickly in order to increase their chances of survival. The energy content of milk is among the highest in mammals, due to a high protein and fat content (Evans & Raga, 2001). As such, milk proteins are good candidates for assessing adaptation in cetaceans. Sequences from several cetacean species are available for different milk proteins from earlier phylogenetic studies (Gatesy *et al.,* 1996; Gatesy, 1998; Gatesy *et al.*, 1999). These include β and κ casein, and α-lactalbumin, genes encoding proteins known to be physiologically important in milk nutrition (Kelleher *et al.*, 2003; Lonnerdal, 2003; Goff, 2010).

Diving adaptations

Cetaceans live exclusively under water, and several species are known to dive to extreme depths in feeding bouts. Such dives require extreme physiological adaptations to cope not only with prolonged times without oxygen, but also with the extreme pressures experienced at those depths (Hoelzel, 2002).

Air breathing vertebrates have a mixture of lipids and proteins in the lungs designated as lung surfactant, whose function is, among others, to prevent the moist alveolar walls from collapsing during normal breathing (Daniels *et al.*, 1998; Daniels & Orgeig, 2001). In diving mammals, the lungs are known to fully collapse under the strong pressures experienced in deep waters (Hoelzel, 2002). Comparison between the lipid and protein composition of the lung surfactant between terrestrial (cattle) and marine mammals (pinnipeds) revealed the existence of differences that might contribute to a more effective prevention of the alveoli from collapsing during deep diving (Miller *et al.*, 2006a; Miller *et al.*, 2006b). Sequence comparison between terrestrial and marine mammals for one of the proteins composing the lung surfactant, SP-C, also found differences between these groups likely to be the result of selection (Foot *et al.*, 2007).

During diving, marine mammals have no access to air and have therefore developed several physiological adaptations to hypoxia. For example, they will lower their heart rate during dive, and reduce the level of blood flow to all tissues except for the brain (Hoelzel, 2002). However, studies have concluded that in spite of these known adaptations, the partial oxygen pressure in cetaceans during diving is still lower than the minimum required to maintain consciousness in other mammals (Williams *et al.*, 2008). A common adaptation to increase

available oxygen in tissues involves the increased expression of oxygen binding globins (Hoelzel, 2002), a process that also occurs in the brain (Williams *et al.*, 2008). Interestingly, the exact composition of such proteins is dependent on whether the animals are deep divers or fast swimmers, indicating that different proteins will have different characteristics suitable for different patterns of hypoxia (Williams *et al.*, 2008). One such protein is the neuroglobin, which is mainly expressed in the brain and is known to have a protective function against hypoxia in brain cells, and whose expression is thought to be controlled by the hypoxia inducible factor (HIF) (Sun *et al.*, 2001; Brunori & Vallone, 2007; Greenberg *et al.*, 2008; Williams *et al.*, 2008).

The HIF is part of a signalling pathway that controls the expression of several different genes in response to reduced oxygen levels. Genes regulated by the HIF are involved in physiological processes such as vascular endothelial growth, erythropoiesis and ATP production (Semenza, 1998). The HIF DNA binding regions were found to be encoded by two different genes, the HIF-1α and the HIF-2α. A study comparing haplotype frequency of the α sub-unit of the HIF2 gene (also known as EPAS1) between high competition athletes and a control group found significant differences between them, indicating that variation in this gene might be important in determining the ability of an individual to cope with activities that demanded increased levels of oxygen (such as prolonged or intense exercise) (Prior *et al.*, 2003; Henderson *et al.,* 2005).

Echolocation

In the ocean, very little light will penetrate roughly beyond 100 metres past the surface, much less in certain turbidity conditions, making vision a less important sense. As such, many cetaceans have developed the use of sound for orientation and prey capture. Additionally, the high pressures cetaceans face during deep diving, might have promoted adaptations to protect sensitive structures like the eyes or the inner ear from damage (Hoelzel, 2002).

In mammals, the capacity to detect and resolve subtle differences in sound frequency, particularly high frequencies, is thought to be due to the cochlear outer hair cells (Randall *et al.,* 2002; Santos-Sacchi, 2003). The morphology of cochlear hair cells in general is quite variable in vertebrates, which is thought to be the result of adaptation to different hearing requirements (Fay & Popper, 2000). Sequence analysis of a gene encoding a transmembrane protein from the solute carrier family (Slc26a5 or prestin) found in the membrane of outer cochlear cells, has shown signs of positive selection in mammals only (Franchini & Elgoyhen, 2006). Further to this, phylogenetic studies of prestin joined all echolocating bats together, in

close relationship with echolocating cetaceans to the exclusion of other non-echolocating species (Li *et al.,* 2008; Li *et al.*, 2010; Liu *et al.*, 2010).

Myocilin is a protein that exhibits high levels of expression in ocular tissues (Gould *et al.,* 2004; Wentz-Hunter *et al.*, 2004; Wentz-Hunter *et al.*, 2004). Its functions are still unclear, but mutations in the gene encoding it have been associated with mild cases of open-angle glaucoma (Kaur *et al.,* 2005; Rose *et al.*, 2007). Open-angle glaucoma (OAG) is a disease caused by the degeneration of the optic nerve, resulting in a progressive loss of field vision eventually leading to blindness (Quigley, 1993). It's thought that although not a consequence of OAG, increased high intra-ocular pressure might contribute to the progress of the disease by increased strain on the optic nerve (Quigley, 1996). A study indicated that increased levels of myocilin expression *per se* do not cause glaucoma, and therefore the production of abnormal proteins must be involved (Gould *et al.*, 2004). Populations that usually feed at night might make more use of echolocation while hunting and such selection against visually impaired animals might be relaxed in comparison with populations that hunt during the day. Alternatively, populations that live or feed in deeper waters might experience a selective pressure for higher intraocular pressure to resist collapse during deep diving.

Thermoregulation

Water has a thermal conductivity 20 times higher than air, and as such maintaining core body temperature imposes a considerable physiological challenge to cetaceans. Blubber is the only isolating mechanism in these animals, and different species of cetaceans are known to have different lipid content and corresponding differences in insulating properties, suggesting adaptation to different water temperatures (Hoelzel, 2002)

A recent study has identified two genes in mammals that are thought to be involved in the formation of adipose tissue, named fat-inducing transcript genes (FIT1 and FIT2). While FIT1 was shown to be expressed mainly on heart and skeletal muscle, FIT2 is mainly expressed in white and brown adipose tissue. Knockout mice lacking these genes exhibited a decreased production of lipid droplets, confirming the importance of these genes in the formation of adipose tissue (Kadereit *et al.*, 2008).

Colouration

Although cetaceans have relatively homogeneous skin colouration across species, some striking differences can be found between taxonomic groups. For example, the beluga whale (*Delphinapterus leucas*) has white skin colour, while most delphinids have dark skin. The common dolphin is one of the only two species of dolphins that exhibit yellow coloured

patches (Folkens *et al.*, 2002). However, a different conspecific morphotype has been described, where the yellow colour is replaced by a black colouration similar to one found on the dorsal side (Perrin *et al.,* 1995; Stockin & Visser, 2005). Colouration patterns in cetaceans are thought to have a predator avoidance function (Pryor & Norris, 1991), but resistance to sunburn by species that spend more time on the surface has also been proposed (Martinez *et al.*, In Press).

Several mutations are known in both MC1R and agouti genes that lead to different proportions of black and yellow for a number of mammal species. Mutations in the MC1R are usually point mutations, whose dominant alleles result in all black colouration (Jackson, 1997; Kijas *et al.*, 1998; Savage *et al.*, 2008), while mutations in agouti are usually related to inserts or deletions of large fragments of DNA, whose dominant alleles result in an all yellow phenotype (Vrieling *et al.*, 1994; Manne *et al.,* 1995). Additionally, other genes are known to influence the intensity of pigmentation, such as TYRP1 (Slominski *et al.*, 2004) where point mutations are related to a change in colouration from black to brown in cattle, mouse and humans (Bell *et al.*, 1995; Berryere *et al.*, 2003; Alonso *et al.,* 2008; Mohanty *et al.*, 2008).

Reproduction

Many cetacean species are thought to have promiscuous and polygynous mating systems. As such, it can be expected that proteins involved in egg-sperm recognition will be under strong selection. In the egg, sperm recognition is mediated by the zona-pellucida, a structure that covers the whole egg surface and is composed of three different glycoproteins, named ZP1, ZP2 and ZP3. There is evidence that, among these, ZP3 is essential in allowing sperm binding, and stopping remaining sperm from binding after the egg is fertilized. Therefore, differences in ZP3 structure act as an effective reproductive barrier between species (Wassarman, 1999). One study comparing ZP3 sequence from different mammalian species found that it showed considerable levels of variation within species and that dN/dS analysis revealed signs of selection (Swanson *et al.*, 2001).

Protamines are proteins that replace histones in binding condensed DNA in sperm (Ammer & Henschen, 1988). In mammals two types of protamine exist, protamine 1 being the most important (Rooney & Zhang, 1999). Studies of this gene in catarrhinean primates showed that the two protamine 1 exons appear to accumulate mutations faster than the single intron in some *taxa*, and that non-synonymous substitutions were significantly more common that synonymous ones, suggesting that natural selection is promoting the evolution of this gene (Rooney & Zhang, 1999; Wyckoff *et al.*, 2000). Several lines of evidence suggested that

variation in the protamine genes might originate variation in sperm head morphology, which results in differences on fertility rates (Rooney & Zhang, 1999; Clark, 2000). Dolphins generally exhibit strong sperm competition as indicated by their large testis size relative to body size (Kenagy & Trombulak, 1986). For example, common dolphins exhibit some level of sexual dimorphism and increased testis size during the mating season (Murphy *et al.,* 2005; Westgate & Read, 2007) while female bottlenose dolphins are known to copulate with multiple males (Connor *et al.*, 1992). As such, diversity levels in protamines may also be high relative to ZP3 in cetaceans.

Immune system

All living organisms are equipped with a set of mechanisms that allows them to stop infection from foreign pathogenic organisms (virus, bacteria, fungi and parasites) that collectively form the immune system. The simplest of those mechanisms consists in the physical barrier that cellular membranes or epithelial surfaces represent to pathogens (Janeway *et al.*, 2001). Pathogens can enter the blood circulation through open wounds, and it can thus be expected that selective pressures would favour more effective blood clotting and scar tissue formation in environments with higher pathogenic load. Fibrinogen is a glycoprotein composed of 3 different units (α, β and γ), and is an essential protein in the blood clotting mechanism, while also being important to the inflammatory response and scar tissue formation (Mosesson *et al.*, 2001). Several mutations in the fibrinogen genes are known to cause blood clotting related problems with symptoms such as chronic bleeding, impaired scaring, and liver cirrhosis (Asselta *et al.,* 2000; Brennan *et al.*, 2001; Matsuda & Sugo, 2001; Neerman-Arbez, 2001; Maghzal *et al.*, 2004). Other problems have also been associated with fibrinogen mutations, such as kidney disease (Brennan *et al.,* 2001; Matsuda & Sugo, 2001) and coronary heart diseases (Green, 2001). Several cetacean sequences are available from earlier phylogenetic studies (Gatesy, 1997; Gatesy, 1998), but evidence for selection on such genes was never investigated.

Once pathogens enter the blood circulation, other more complex molecular cellular mechanisms exist to stop infection. These can be divided in two groups according to specific characteristics of how they fight infection, called the innate immune system and the adaptive immune system. The innate immune system reacts to molecular and cellular structures found in a wide range of foreign pathogens, while the adaptive immune system provides more effective and long-lasting protection by identifying and targeting unique characteristics of specific pathogens and by remembering them for future infections. In order for the adaptive

immune system to be effective, lymphocytes (the adaptive immune system effector cells) must be able to discriminate between different antigens. There are two different classes of lymphocytes, B-cells and T-cells. One of the main functions of T-cells is to recognize antigens, and lead them to phagocytic cells such as B-cells, macrophages or dendritic cells. In addition, T-cells can also eliminate virus infected cells.

The Major Histocompatibility Complex (MHC) is a family of genes that encode proteins whose general function is to bind pathogenic proteins and present them on the surface of infected cells so that they can be recognized by T-cells (Janeway *et al.*, 2001). Within the MHC, genes are classified into two categories, named class I and class II, according to their specific function (Janeway *et al.*, 2001). MHC genes were the first to be shown to be under selection through dN/dS analysis (Hughes & Nei, 1988; Hughes & Nei, 1989), and several lines of evidence strongly suggest that diversity in these genes is actively maintained by selective pressures imposed by different pathogenic loads (Hughes & Yeager, 1998). Several studies have also shown evidence for selection on a population level in several species of mammals (Bernatchez, 2003), including some species of cetaceans for the MHC class II HLA-DQβ *locus* (Munguia-Vega *et al.,* 2007; Vassilakos *et al.*, 2009; Yang *et al.*, 2010).

Toll-like receptors (TLR) constitute another class of antigen receptors similar in function to the MHC, that activate phagocytes and tissue dendritic cells (Janeway *et al.*, 2001). TLR's are transmembrane proteins that will recognize different structures characteristic of infectious bacteria, such as the bacterial cell wall (Janeway *et al.*, 2001). Different TLR's will be specific to different structures, with TLR3 in particular recognizing double stranded RNA produced during viral reproduction (Alexopoulou *et al.*, 2001). As such, TLR3 has a very similar pathogen recognition system as the DQβ *locus* but is specific to viral infections.

3.2. OBJECTIVES

In this study, a candidate marker approach will be used to investigate adaptation in cetaceans, both in the order as a whole and at a population level. Appropriate markers to use in the population level analysis will be identified by carrying out dN/dS tests in several functionally relevant genes in mammals and cetaceans where enough sequence are available. The objective is to identify regions of the genome that have evolved and differentiated rapidly in response to the transition from land to sea in the cetacean lineage, and assess if such patterns can also be observed at a population level as evidence that adaptation promotes differentiation in such organisms. This will be done under the assumption that markers that

show signals of positive selection in higher taxonomic levels are more likely to also exhibit signals of selection at a population level, an assumption that will be evaluated in light of the results. The population level analysis will focus on European common dolphins. Patterns of genetic structure on neutral markers are well described in this population (see Chapter 2 of this work and references therein), and the strong environmental cline that can be observed between the Mediterranean and the North Atlantic (Pinet, 2003), can be used to assess correlations between environmental differences and any potential patterns observed.

3.3. METHODS

3.3.1. Candidate markers choice

Choice of candidate markers was based on a combination of factors, namely, knowledge of a physiologically relevant function (discussed in the introduction), detection of selection along the mammalian class and availability of cetacean sequences to carry out selection analysis. Throughout the following sections of this chapter, different markers will be represented by their GenBank abbreviations, indicated in Table 3.1 (page 76).

Markers that do not have a well described functional relevance will be difficult to interpret in the light of positive (or negative) results, while markers that have evolved neutrally, or have been under purifying selection over a wide taxonomic range, are less likely to show evidence of local adaptation. As such, to determine the applicability of candidate markers for their use in population studies of the common dolphin, the potential for detecting signals of selection was investigated in mammals in general, and if enough sequences were available, in cetaceans in particular. Studies of selection have previously been done in several of the markers analysed here, with varying degrees of species representation among mammals. These will still be included in the present study for the sake of consistency in the mammalian groups sampled between markers, and also to act as positive controls for which selection has been confirmed independently. The only exceptions are protamine and ZP3 as both these markers have been shown to be under selection on a large range of mammalian groups. As a negative control, one gene was included whose physiological function would be difficult to integrate in an environmental adaptation context. Calcium/calmodulin-dependent protein kinase II is an enzyme primarily expressed in neurons that is activated by high levels of Ca+ and active calmodulin (Lin *et al.*, 1987; Carlton, 2002; Djakovic *et al.*, 2009). Its exact physiological functions are unclear, but variation in the gene coding this enzyme has been related to pain sensitivity and neurological conditions such as depression (Brüggemann *et al.,* 2000; Novak *et*

Table 3.1. GenBank abbreviations used in the chapter for each functional gene analysed.

GenBank Code	Gene
CAMKA2	Ca/calmodulin-dependent protein kinase II, α chain
AQP1	Aquaporin 1
AQP6	Aquaporin 6
ASIP	Agouti Signalling Protein
CSN2	α-casein
CSN3	κ-casein
FGG	γ-fibrinogen
FIT1	Fat inducing transcript 1
FIT2	Fat inducing transcript 2
HIF1	Hypoxia inducible factor 1
HIF2	Hypoxia inducible factor 2
LALBA	α-lactalbumin
MYOC	Myocilin
TLR3	Toll-like receptor 3
MC1R	Melanocortin receptor 1
TYRP1	Tyrosinase-related protein 1
UT-A2	Urea-transporter 2, α chain
NGB	Neuroglobin
Prestin	Prestin
SP-C	Lung surfactant protein C
Protamine	Protamine 1
ZP3	Zona Pellucida 3

al., 2006; Chen *et al.*, 2010). Although the exact function is unknown, studies so far have not revealed any function that could potentially be thought of as having any adaptive value for marine mammals.

3.3.2. Data collection/sequence alignment

Mammalian sequence data was retrieved from the ORTHOMAM online database (Ranwez *et al.*, 2007). ORTHOMAM uses data from available mammalian genomes to create alignments of orthologous genes based on the well annotated genomes of human, mouse and dog. Currently, data for up to 36 different species of mammals are available. However, because different genes will have different species coverage, an attempt was made to include the same taxonomic groups in each alignment, but not necessarily the same species. The bottlenose dolphin (*Tursiops truncatus*) was present in all ORTHOMAM alignments and was always included to test for branch specific selection. Because the proportion of missing data can be variable for certain species, additional searches were performed in GenBank to complement species with a high proportion of missing data. Species known to be under selection for a particular gene, were removed from the alignment as their presence in the database might give the illusion of a stronger selection pattern across mammals. For example the cow (*Bos*) sequence was removed from all milk protein alignments.

Additional cetacean sequence data was obtained from GenBank for each gene obtained in the previous analysis, when available. Species representation within Cetacea was highly variable, with most groups being available for some markers, while only a few species being available for others. In markers where a good representation was available, alignments containing only cetacean species and outgroup was included. Otherwise, cetacean sequences were added to the mammalian alignment. Sequences from other species were also added as relevant in particular cases. All the alignments were done using either CLUSTALW (Thompson *et al.*, 1994), MUSCLE (Edgar, 2004) or MAFFT (Katoh *et al.*, 2002) algorithm as appropriate. Because complete mRNA's were not available for all the species marker combinations (either only partial exons or sequences including exons and introns), alignments tended to be complex and CLUSTALW frequently failed. In cases where complete mRNA's were available for all species, MUSCLE and MAFFT will often introduce excessive alignment gaps in areas of high diversity. All alignments were implemented in the software package GENEIOUS (Drummond *et al.*, 2010).

3.3.3. Detection of selection

Sequence model evolution for each alignment was determined using TOPALI V2 (Milne *et al.*, 2008). Phylogenetic trees for use in the maximum likelihood dN/dS analysis were produced using the PHYML algorithm (Guindon & Gascuel, 2003) implemented in the program TOPALI V2 (Milne *et al.*, 2008). Robustness of the trees was assessed through bootstrap with 1,000 replicates. Independent trees were constructed using the MRBAYES algorithm (Huelsenbeck & Ronquist, 2001) implemented in the software package GENEIOUS (Drummond *et al.*, 2010). Four independent chains were run for 1,100,000 replicates with 110,000 burnin replicates, and a sampling frequency of 200 replicates.

Signals of selection were detected using the maximum likelihood dN/dS approach implemented in PAML (Yang, 2007) integrated in the software package TOPALI V2 (Milne *et al.*, 2008). Instead of pairwise dN/dS analysis, PAML compares statistical distributions among sites that allow or not for positive selection to occur. These models are then compared for significance using a likelihood ratio test with a χ^2 distribution (Yang, 2007). Three pairs of models were compared independently, based on their well known robustness in detecting positive selection (Wong *et al.,* 2004; Yang, 2007; Yang, 2009): M0 vs. M3 – M0 is the simplest model and assumes a single value of dN/dS (ω in the PAML package) common to all sites (Goldman & Yang, 1994), while M3 allows for 3 distinct values of ω across sites, each with distinct proportions (p) (Yang *et al.*, 2000); M1a vs. M2a – M1a represents a nearly neutral model with two classes of ω where one (ω0) is allowed to vary from 0 to 1 and the other (ω1) is fixed at 1, while M2a represents positive selection by adding an extra class where ω is allowed to have any value above 0 (ω2) (Nielsen & Yang, 1998; Wong *et al.*, 2004; Yang *et al.*, 2005); M7 vs. M8 – M7 assumes a β distribution of ω across sites where ω is only allowed to vary between 0 and 1, while M8 allows for an extra proportion of sites to adopt any value of ω above 0 (ω2) (Yang *et al.*, 2000). From these tests, only M1a vs. M2a and M7 vs. M8 are real tests of positive selection, whereas M0 vs. M3 only test for heterogeneity of ω across sites (Anisimova *et al.*, 2001). Therefore, models M1a, M2a, M7 and M8 were tested multiple times with different initial values to avoid local optima, as suggested in (Wong *et al.,* 2004; Yang, 2009). Sites under positive selection were identified using the empirical Bayesian method of (Yang *et al.*, 2005).

Branch model analyses were also carried out. In the mammalian alignments, branch model analyses were done by comparing a tree with constant dN/dS to a tree where the branch leading to the bottlenose dolphin was allowed to vary (Yang & Nielsen, 2002). In the cetacean

alignments, branch analysis was done by comparing hypotheses relevant to each marker (discussed below). Candidate markers for the population level analysis, where selected based on evidence for selection from the phylogenetic analysis, availability of template sequences to design PCR primers, exon/intron complexity of the marker (genes with numerous small exons separated by large introns are difficult to amplify fully using PCR), and known physiological relevance.

For the population level analysis, genotype frequencies for each successfully screened non-synonymous mutation was counted, and deviations from Hardy-Weinberg equilibrium calculated using exact tests with 1,000,000 iterations and 100,000 of dememorization steps. Pairwise Fst between locations was calculated, with significance assessed through 1,000 permutations. All calculations implemented in the software ARLEQUIN (Excoffier, 2005).

3.3.4. Laboratory procedures

For each selected candidate gene, primers were designed using the PRIMER3 (Rozen & Skaletsky, 2000) algorithm as implemented in GENEIOUS (Drummond *et al.*, 2010). Primers were targeted at conserved regions of an alignment including either cetacean or arctiodactyl sequences, depending on availability. Seven markers were successfully amplified and sequenced in the common dolphin (Table 3.2, page 80). Amplification was carried out using Phusion Taq polymerase (Finnzymes) because of its proofreading capabilities and fast PCR times, allowing for quick optimization of PCR conditions. DNA was placed in a tube containing 1X High Fidelity Buffer (Finnzymes), variable concentrations of dNTP's (Table 3.2, page 80), 0.6 μM of each primer and 0.2 U of Phusion Taq (Finnzymes). PCR conditions followed a general protocol, with only variations in the dNTP and DNA concentration, annealing temperature and number of cycles. General conditions involved initial denaturation at 98°C for 3 minutes, variable number of cycles (Table 3.2, page 80) of denaturation at 98°C for 10 seconds, annealing at variable temperatures (Table 3.2, page 80) for 30 seconds and extension at 72°C for 30 seconds. One last extension step at 72°C for 7 minutes was included in all markers. All primers were designed in this study.

Population level analyses were made using the same set of samples as used in Chapter 2 (Figures 2.1 & 2.2, pages 33 & 35). For each successfully amplified candidate gene (Table 3.2, page 80), variation was screened by sequencing 12 individuals sampled along the Portuguese coast, 2 for each sampling location as indicated in Chapter 2. Heterozygous positions were confirmed through cloning. Cloning was done using the CloneJet PCR Cloning Kit protocol

Table 3.2. Primer sequence and PCR conditions for all successfully amplified candidate markers. MYOC required a touchdown protocol, hence the inclusion of multiple annealing temperatures. Agarose gel pictures available in Appendix 3.3, page 170.

Marker	Primers	Size (bp)	[DNTPs]	DNA	Ta(°C)	Cycles
Protamine 1	5'- ACT TGC TCA CAG GTT GGC -3' 5'- GGA GTG TGG TGG TCT TGC -3'	299	0.3 mM	1 µl	60	35
CSN2 exon 7	5'- GTC TAT TCC TAC ACT GGG -3' 5'- TGG AAC AGC AGA AGG G -3'	355	0.3 mM	0.2 µl	59	30
SP-C exon 2	5'- GAA GCC TTC TCC GAT CTC C -3' 5'- TCA TGT AAA GAG CCA TGA GCA G -3'	247	0.2 mM	1 µl	62	30
UT-A2 exon 1	5'- CTT GCC CGT CTC TTG TC -3' 5'- TCT GTG CAG CGT GTT TC -3'	207	0.2 mM	1 µl	64	30
MYOC exon 3	5'- GGA TGT GGA GAA CTC ATT TGG -3' 5'- TGA AGT TGT CCC AGG CAA AG -3'	683	0.2 mM	1 µl	65-59 59	20 10
AQP1 exon 1	5'- CCG CCA GGG TCC TAT AAA T-3' 5'- TCC TCT CTG TTC CTC GGT GT -3'	587	0.1 mM	1 µl	63	30
TYRP1 exon 1	5'- CAA GGA AAT CAG TGG GAA GG -3' 5'- CCT TTA TCC TAA TGG AGT TTT GG -3'	272	0.1 mM	1 µl	61	30
TYRP1 exon 2	5'- TCA TCT TGC TTT TTC CTT TTC A -3' 5'- CAT CCA TTC ACA CCC CCA CTT -3'	471	0.2 mM	1 µl	62	30
TYRP1 exon 4	5'- ATG CCA GGG AGT AAA CCA AG -3' 5'- TTC AGG CAG CAC ATT TAA TCA C -3'	327	0.1 mM	1 µl	62	35
TYRP1 exon 5	5'- TCA CCG TTT CCC ATC TTT TC -3' 5'- CGG CCC TAT TCA CAT CAA C -3'	255	0.2 mM	1 µl	59	35
TYRP1 exon 6	5'- GGC CTG ACA AGC TTA GGA AA -3' 5'- TTG CTG AGC CTG CAA AAA G -3'	289	0.1 mM	1 µl	62	35
TYRP1 exon 7	5'- TTT TAA AGT TAC CCT TGA ATA CTT TGG -3' 5'- GGG TGA CAA ATT TGC TTT CG -3'	269	0.1 mM	1 µl	62	35
TYRP1 exon 8	5'- TTG GGT GGC GAC TGT TTT -3' 5'- TGT GAG TCT GTT TCT GCT TCG T -3'	615	0.2 mM	0.5 µl	63	30

(Fermentas) according to the manufacturer's instructions, with 4 clones sequenced for each sample using the forward primer pJET 1.2 included in the CloneJet cloning kit. Because these sequences were used to test for selection at a population level, they were not added to the cetacean phylogenies.

For the markers where variation was found, non-synonymous point mutations were identified and screened using restriction enzyme based method. Screening was performed for the Irish and Greek populations, located at the edges of the European distribution of the species, as well as for the Portuguese population, located in the transition zone between the Mediterranean Sea and the Atlantic Ocean (total number and location of samples analysed provided in the results section). Appropriate restriction enzymes were identified using the online software SNP-RFLPING (Chang *et al.*, 2010), and the online software RESTRICTIONMAPPER (http://www.restrictionmapper.org/) was used to predict cutting patterns for the different alleles. Restriction digestion was done in a PCR machine according to the manufacturer's indication, and results were checked in a 1.5% agarose gel stained with ethydium bromide, later photographed on a UV transluminator. To confirm correct genotype identification for each mutation, the restriction digestion process was repeated for 20 randomly chosen samples, and a subset of 4 samples was re-sequenced directly.

The exon 2 of the MHC DQβ1 *locus* was amplified using the primers from (Tsuji *et al.*, 1993) following the PCR conditions described in (Vassilakos *et al.*, 2009). Variation was assessed using an SSCP protocol based on the one described in (Vassilakos *et al.*, 2009). For each sample, 2 μl of PCR product was mixed with 2 μl denaturing loading buffer following the formula described in (Vassilakos *et al.*, 2009). Samples with loading buffer were denatured at 98°C for 5 minutes and placed on ice for 3 minutes straight after. They were then run on a Sequi-Gen GT vertical electrophoresis system (BioRad) in a 6% (v/v) 37.5:1 acrylamide:bis-acrylamide gel with 10% (v/v) Glycerol in 1X TBE. Gels were run for 5 hours at constant 40 W and room temperature, after which they were stained for a minimum of 30 minutes with GelStar Nucleic Acid Gel Stain (Lonza, Inc.) and photographed in a BioRad UV transluminator.

Unique alleles were extracted from the acrylamide gel using the following protocol: single stranded bands were extracted from the gel and placed in one 1.5 mL microcentrifuge tube each; gel slices were eluted in autoclaved water at 37°C overnight; samples were then centrifuged for 1 minute at 13,000 rpm, and the supernatant was placed in a new tube using a micropipette. Extracted sequences were then reamplified by placing 1 μl of the resulting

template following the same protocol as before (from (Vassilakos *et al.*, 2009)). The resulting product was then purified using Qiaquick PCR Purification Kit (Qiagen), and sequenced on an ABI 3730 automatic sequencer using both forward and reverse primers.

3.3.5. MHC Data Analysis

For the analysis of MHC DQβ1 variation along the European coast, several genetic differentiation statistics between sampling locations were calculated using DNASP V5.1 (Librado & Rozas, 2009). Pairwise Fst between locations and Analysis of Molecular Variance (AMOVA) were calculated using the program ARLEQUIN (Excoffier, 2005). Significance of pairwise Fst comparisons was assessed with 1,000 permutations as implemented in the software ARLEQUIN (Excoffier, 2005). A median-joining network was constructed with maximum parsimony processing using the software NETWORK (Bandelt, 1999; Bandelt, 2000).

It is well described that differences in the ability of MHC DR *locus* to bind pathogenic agents are dependent on the combined charge profiles of specific amino acid positions on the pocket 4 region (residues 70, 71 and 74 of the β chain)(Ou *et al.*, 1998). Given that DR and DQ *loci* are thought to have originated by duplication of an ancestral gene (Kumanovics, 2003), and that they retain similar antigen peptide binding functions (Janeway *et al.*, 2001), the criteria of (Ou *et al.*, 1998) can serve as a template to investigate functional differences in the DQ *locus*. As such, differences in the frequency of charge profiles in those positions between populations might reflect selection for functional differences. Differences in the frequency of alleles with different charge profiles in the pocket 4 region were assessed manually following the criteria described in (Ou *et al.*, 1998). Statistical significance of the differences found was assessed through contingency tables as implemented in the software RXC (http:// www.marksgeneticsoftware.net). dN/dS analysis was carried out following the same protocol as described earlier, both for all generated sequences, and for each sampling location.

3.4. RESULTS

3.4.1. Mammalian datasets

All the alignments showed considerable length variation between species, with several sequences exhibiting both end gaps and insertions/deletions (indels) (Table 3.3, page 83). Phylogenetic analysis showed considerable variation in the resolution of different markers, with some markers exhibiting particularly low resolution at the basal nodes (ASIP, CSN3, FIT2, SP-C, TYRP1). Tree topologies were essentially consistent between markers, especially

Table 3.3. Mammalian alignments used in dN/dS analysis. Models identified as significantly better supported than neutrality are indicated. More details regarding the dN/dS analysis in mammals can be found in Appendix 3.1, page 144.

Marker	N	Sequence Length	Pairwise Identity	Gamma shape	Sig-PS-Models
CAMKA2	23	1050-1467	78.2%	0.308	M3
AQP1	21	531-636	84.4%	0.514	M3, M2a, M8
AQP6	20	522-840	79.5%	0.502	M3, M2a, M8
ASIP	26	318-411	78%	1.255	M3
CSN2	24	564-750	61.6%	2.569	M3, M2a, M8
CSN3	25	321-561	64.4%	3.278	M3, M2a, M8
FGG	25	798-1341	80.1%	0.553	M3, M8
FIT1	24	588-885	84.6%	0.344	M3, M8
FIT2	26	651-786	82.8%	0.466	M3
HIF1	24	1899-2499	83.7%	0.526	M3, M8
HIF2	22	2142-2625	79.8%	0.468	M3
LALBA	21	366-429	78.2%	0.971	M3, M2a, M8
MYOC	23	1086-1512	75%	0.556	M3, M2a, M8
TLR3	23	2271-2724	80.3%	1.297	M3, M2a, M8
TYRP1	24	1203-1617	83.7%	1.075	M3, M8
UT-A2	23	2562-2772	78.7%	0.417	M3, M8
NGB	20	366-468	83.1%	0.517	M3
Prestin	24	1344-2247	77.8%	0.387	M3, M2a, M8
SP-C	23	450-627	77.4%	0.615	M3, M8

for primates. However, some taxa appeared to jump position considerably, especially the horse (*Equus*), bats (*Myotis* & *Pteropus*) and the hedgehog (*Erinaceus*). Interestingly, these taxa had a tendency to group in closely related clades. The position of the bottlenose dolphin (*Tursiops truncatus*) was always consistent, grouping with either cow (*Bos*) or pig (*Sus*), with the notable exception of NGB where it falls basal to all eutherian mammals (Appendix 3.1, page 157).

All 21 markers showed support for heterogeneity of ω among sites (M3), but only 14 markers showed evidence for positive selection (M2a or M8) (Table 3.3, page 83). From these, only 8 had significant statistical support for both M2a and M8 positive selection models. From the 5 markers showing support for only the M3 models, the largest proportion of sites had ω values very close to 0, suggesting they are under purifying selection (Appendix 3.1, page 144 - 150). In the branch analysis, only CAMKA2 and FGG showed significant results for the bottlenose dolphin branch.

3.4.2. Cetacean datasets

Good representation for cetaceans was only available for the markers CSN2 (partial exon 7), CSN3 (partial exon 4), LALBA (exons 1 to 3), Protamine 1 (exon 1 & 2), ZP3 (partial exons 6 & 7) and MC1R (full exon). Therefore, only those markers were analysed for cetaceans (including some outgroup sequences). For the other markers (FGG, SP-C, UT-A2 and Prestin), due to weak cetacean representation, available sequences were added to the mammalian dataset. For Prestin, sequences of bats available from (Li *et al.,* 2010; Liu *et al.*, 2010) were added for comparison, and for SP-C, pinniped sequences available from (Foot *et al.*, 2007) were added for comparison. These were used as positive controls for the detection of selection, as these markers have been shown to be under selection in previous studies (Foot *et al.,* 2007; Li *et al.*, 2010; Liu *et al.*, 2010).

Resolution in the cetacean phylogenetic trees was considerably reduced compared to the mammalian datasets, owing to the high degree of similarity among taxa, especially in LALBA, MC1R and ZP3 where pairwise identity was higher than 95%. Topologies within cetaceans were generally consistent with previous studies, with some notable exceptions. In particular, baleen whales are polyphyletic for CSN3, and the sperm whale (*Physeter macrocephalus*) is basal to all cetaceans in ZP3 (Appendix 3.2, pages 165 & 169 respectively).

From all the alignments including only cetaceans and outgroups, only LALBA and MC1R showed no signs of selection, while MC1R showed evidence for ω heterogeneity among sites (M3)(Table 3.4, page 85). All the other markers showed strong signs of positive selection with the exception of UT-A2 that showed support for positive selection only for the M8 model (Table 3.4, page 85), consistent with the previous analysis (Table 3.3, page 83).

Table 3.4. Cetacean alignments used in dN/dS analysis. Models identified as significantly better supported than neutrality are indicated. More details regarding the dN/dS analysis in cetaceans can be found in Appendix 3.2, page 161.

Marker	N	Sequence Length	Pairwise Identity	Gamma shape	Sig-PS-Models
CSN2	28	343-424	92.4%	1.124	M3, M2a, M8
CSN3	11	196-435	89.5%	n/a	M3, M2a, M8
FGG	31	225-231	79.1%	1.077	M3, M2a, M8
LALBA	35	210-306	96.1%	0.650	-
MC1R	30	933-936	96.2%	0.397	M3
SP-C	33	102-108	86.2%	0.362	M3, M2a, M8
UT-A2	24	1002-1191	87.3%	0.331	M3, M8
Prestin	41	2022-2241	87.5%	0.688	M3, M2a, M8
Protamine 1	35	141-156	86%	0.891	M3, M2a, M8
ZP3	21	128-220	95.9%	0.702	M3, M2a, M8

3.4.3. Variation along the Portuguese coast

For 12 individuals sampled along the Portuguese coast, 5 markers showed variation within exons. From those, 3 (CSN2; TYRP1 exon 5; TYRP1 exon8) had non-synonymous mutations. Notably, in 2 cases (CSN2 and TYRP1 exon 5) all the mutations found in exons were non-synonymous. Further to this, CSN2 stands out as having an unusually high number of mutations compared to other markers, all of them non-synonymous (Table 3.5, this page).

Table 3.5. Number of mutations, synonymous and non-synonymous, found by screening 12 individuals sampled along the Portuguese coast.

Marker	Mutations	Synonymous	Non-Synonymous
Protamine 1	0	-	-
CSN2	6	0	6
SP-C	0	-	-
UT-A2	0	-	-
MYOC	2	2	0
AQP1	0	-	-
TYRP1 exon 1	1	1	0
TYRP1 exon 2	0	-	-
TYRP1 exon 4	0	-	-
TYRP1 exon 5	1	0	1
TYRP1 exon 6	0	-	-
TYRP1 exon 7	0	-	-
TYRP1 exon 8	3	2	1

From the identified non-synonymous substitutions, 3 were successfully screened for samples from the European coast using restriction enzymes, one in TYRP1 exon 8 (TP8-114 A/C using the enzyme Hin1II) and two in CSN2 (CSN2-105 A/G using the enzyme Hin6I; CSN2-255 G/T using the enzyme BseGI). In all cases, mostly only one variant of each mutation was found in the homozygous state, while the other, when found, was mostly in the heterozygous state. Heterozygous individuals were always found in very low frequency (Table 3.6, page 87). Most populations did not significantly deviate from Hardy-Weinberg expectations, with the exception of Greece and Ireland for CSN-255 G/T point mutation (Table 3.6, page 87). These were also the only cases were pairwise Fst values were positive and significant (Table 3.7, page 88).

Table 3.6. Genotype frequency and the total number of individuals genotyped in different locations along the European coast, for each non-synonymous point mutation analyzed. Rows marked with a star represent populations that were found to deviate from Hardy-Weinberg equilibrium for that particular point mutation.

TP8-114	AA	AC	CC	Total
Greece	15	2	0	17
Portimão	44	5	0	49
Sagres	33	5	0	38
Sines	19	3	0	22
Peniche	25	3	0	28
Figueira	23	3	0	26
Porto	19	4	0	23
Ireland	16	1	0	17
CSN2-105	**AA**	**AG**	**GG**	**Total**
Greece	16	2	0	18
Portimão	49	4	0	53
Sagres	33	5	0	38
Sines	24	1	0	25
Peniche	22	2	0	24
Figueira	22	1	0	23
Porto	19	1	0	20
Ireland	17	1	0	18
CSN2-255	**GG**	**GT**	**TT**	**Total**
Greece*	6	10	0	16
Portimão	47	6	0	53
Sagres	35	3	0	38
Sines	22	3	0	25
Peniche	23	1	0	24
Figueira	23	0	0	23
Porto	17	3	0	20
Ireland*	14	19	0	33

Table 3.7. Pairwise Fst comparisons between European locations for all non-synonymous point mutations. Fst values are above the diagonal. Significant P-values below the diagonal.

TP8-114	Greece	Portimão	Sagres	Sines	Peniche	Figueira	Porto	Ireland
Greece	0	-0.01959	-0.02133	-0.02602	-0.02394	-0.02492	-0.02041	-0.01979
Portimão	n.s.	0	-0.00978	-0.01392	-0.01416	-0.01449	-0.00508	-0.01471
Sagres	n.s.	n.s.	0	-0.01822	-0.01442	-0.01589	-0.01442	-0.00869
Sines	n.s.	n.s.	n.s.	0	-0.01878	-0.02047	-0.02023	-0.01102
Peniche	n.s.	n.s.	n.s.	n.s.	0	-0.01873	-0.01134	-0.01718
Figueira	n.s.	n.s.	n.s.	n.s.	n.s.	0	-0.01434	-0.01572
Porto	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0	0.00236
Ireland	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0
CSN2-105	**Greece**	**Portimão**	**Sagres**	**Sines**	**Peniche**	**Figueira**	**Porto**	**Ireland**
Greece	0	-0.01495	-0.01998	-0.00541	-0.02273	-0.00888	-0.01453	-0.01868
Portimão	n.s.	0	-0.00299	-0.0098	-0.01516	-0.01175	-0.01502	-0.01747
Sagres	n.s.	n.s.	0	0.00638	-0.01174	0.00315	-0.00229	-0.00643
Sines	n.s.	n.s.	n.s.	0	-0.0128	-0.02124	-0.02243	-0.02311
Peniche	n.s.	n.s.	n.s.	n.s.	0	-0.0152	-0.01915	-0.02204
Figueira	n.s.	n.s.	n.s.	n.s.	n.s.	0	-0.02369	-0.0246
Porto	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0	-0.02695
Ireland	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0
CSN2-155	**Greece**	**Portimão**	**Sagres**	**Sines**	**Peniche**	**Figueira**	**Porto**	**Ireland**
Greece	0	0.25087	0.27679	0.19187	0.28386	0.33918	0.14999	-0.02226
Portimão	0.0	0	-0.00827	-0.01484	-0.00049	0.02616	-0.01454	0.18342
Sagres	0.0	n.s.	0	-0.01214	-0.01164	0.01477	-0.0064	0.19938
Sines	0.0	n.s.	n.s.	0	-0.00104	0.03766	-0.02117	0.14049
Peniche	0.0	n.s.	n.s.	n.s.	0	-0.0009	0.01058	0.20461
Figueira	0.0	n.s.	n.s.	n.s.	n.s.	0	0.05817	0.24152
Porto	0.027	n.s.	n.s.	n.s.	n.s.	n.s.	0	0.11067
Ireland	n.s.	0.0	0.0	0.0	0.0.	0.0	0.036	0

3.4.4. MHC diversity

76 samples were successfully genotyped using SSCP, 13 from Greece, 27 from Ireland and 36 from Portugal. 35 unique alleles were identified through direct sequencing of single stranded DNA extracted from the SSCP gel.

Median joining network showed no correspondence between location and specific haplotypes (although each location had unique haplotypes), and a generally high level of diversity between them (Figure 3.1, this page).

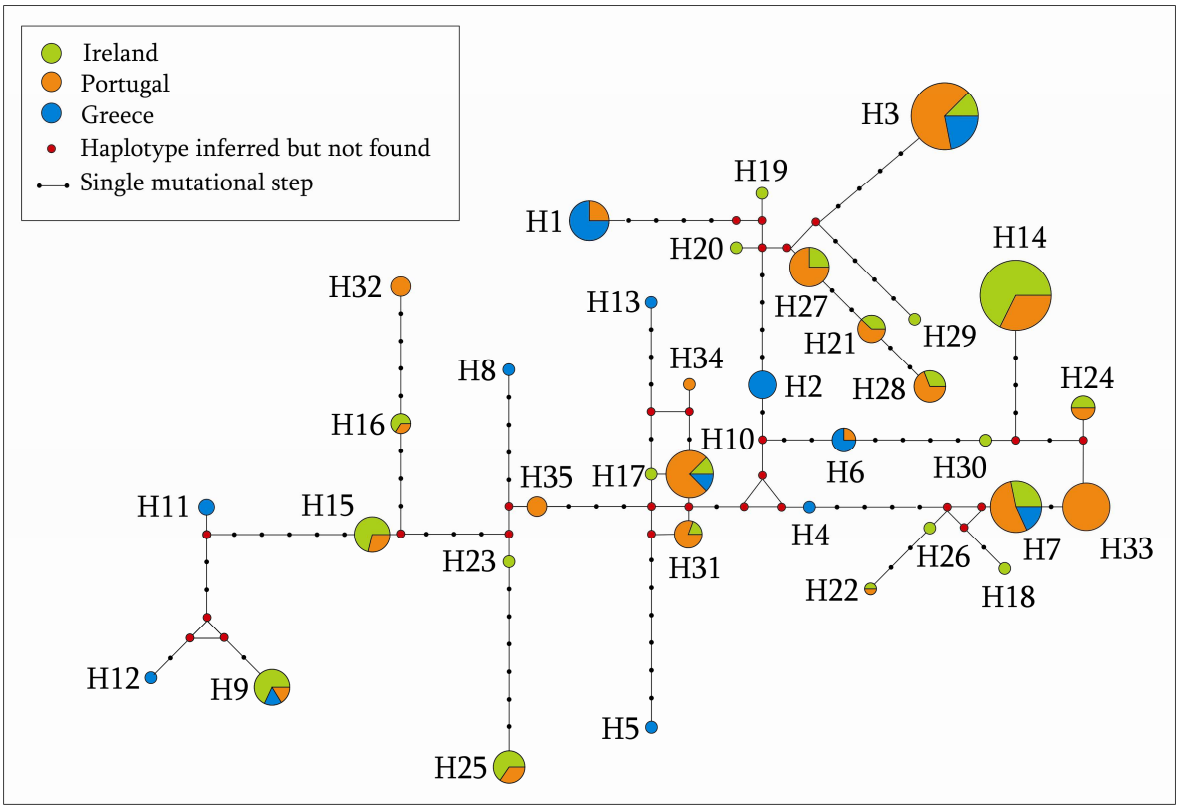


Figure 3.1. Median joining network of DQβ1 exon 2 haplotypes. Size of the circles is proportional to the number of chromosomes in each haplotype; black circles represent single point mutations separating haplotypes.

Diversity indexes within each location showed generally high levels of diversity and essentially similar in each location (Table 3.8, this page).

Table 3.8. Genetic diversity of the DQβ1 exon 2 *locus* within each European location. N-number of sequences analysed; Hn-number of different haplotypes; Hd-haplotype diversity (Nei, 1987); K-average number of nucleotide differences (Tajima, 1983); Pi-nucleotide diversity (Nei, 1987).

Location	N	Hn	Hd	K	Pi
Ireland	54	22	0.93	11.35	0.053
Portugal	72	20	0.93	10.41	0.049
Greece	26	12	0.92	11.98	0.056
Total	152	22	0.93	11.35	0.053

All pairwise Fst comparisons between locations were significant, with the highest Fst values found in the comparison between Greece and Ireland and the lowest between Ireland and Portugal (Table 3.9, this page). AMOVA revealed, however, that most of the variation was distributed within populations (Table 3.10, this page).

Table 3.9. Pairwise Fst comparison between European locations. Fst values above the diagonal; significant comparisons at 0.05 level indicated by an asterisk below the diagonal

Population	Ireland	Portugal	Greece
Ireland	-	0.03514	0.10177
Portugal	*	-	0.05283
Greece	*	*	-

Table 3.10. AMOVA results for pairwise comparisons between European locations and for a comparison including all three European locations. d.f.—degrees of freedom.

Comparison	Source	d.f.	Sum of Squares	Variance Component	Percentage of Variation
Ireland vs. Portugal	Among Populations	1	17.6	0.2	3.51
	Within Populations	124	670.4	5.4	96.49
Portugal vs. Greece	Among Populations	1	16.9	0.3	5.28
	Within Populations	96	519.4	5.4	94.72
Ireland vs. Greece	Among Populations	1	28.7	0.6	10.18
	Within Populations	78	450.4	5.8	89.82
All Locations	Among Populations	2	40.6	0.3	5.4
	Within Populations	149	820.1	5.5	94.6

Although tests for isolation by distance are not possible with only 3 locations, the Fst values are proportional to the distance by sea between each location. The same pattern is observed for several other genetic divergence statistics between locations (Table 3.11, this page).

Table 3.11. Pairwise comparison of different genetic diversity statistics between locations as implemented in the software DNASP (Librado & Rozas, 2009). Comparisons are ordered according to increasing distance by sea from top to bottom.

Comparison	Hs	Ks	Kxy	Gst	DeltaSt	GammaSt	Nst	Dxy	Da
Ireland vs. Portugal	0.93	10.81	11.27	0.014	0.0013	0.02552	0.036	0.053	0.0018
Portugal vs. Greece	0.93	10.82	11.78	0.020	0.00162	0.03158	0.049	0.055	0.0027
Ireland vs. Greece	0.92	11.55	12.96	0.029	0.00336	0.05998	0.101	0.060	0.0061

dN/dS analysis showed strong signals of positive selection for all the sampling locations, with similar result being obtained for the dataset including all sequences (Table 3.12, pages 92 -93).

Comparisons of the frequency of different charge profiles on the DQβ exon2 pocket 4 revealed that haplotypes with no charge were more common than haplotypes with either positive or negative charged profiles. Negatively charged haplotypes showed a decrease in frequency going from Ireland to Greece, while positively charged haplotype frequencies showed an increasing trend (Figure 3.2, this page).

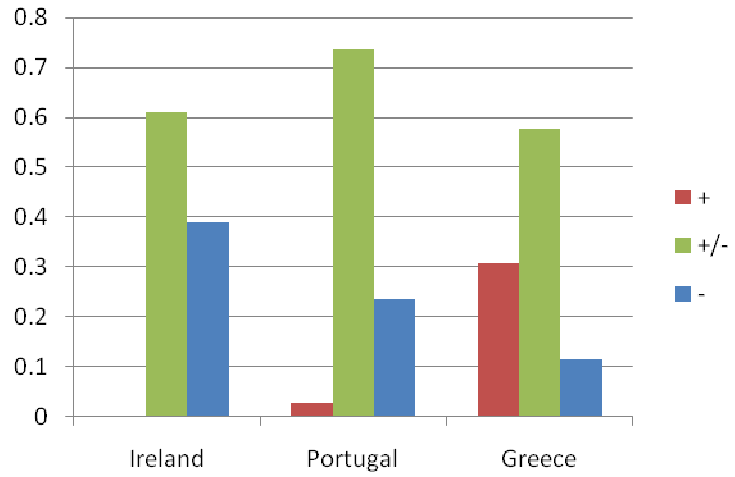


Figure 3.2. Frequency of differently charged haplotypes classified according to (Ou *et al.*, 1998) within each European location

Contingency table analysis showed that frequencies of all charge profile classes were independent when all locations where compared. However, when locations were compared pairwise, only the comparisons involving Greece were significant (Table 3.13, this page).

Table 3.13. p values and standard errors for each contingency table of the frequency of charged haplotypes classified according to (Ou *et al.*, 1998) for pairwise comparison between European locations.

Comparison	p-value	Standard Error
Ireland vs. Portugal	0.096920	0.006646
Portugal vs. Greece	0.000280	0.000130
Ireland vs. Greece	0.000100	0.000100

Table 3.12. dN/dS results for the DQβ1 exon 2 *locus* on European common dolphins. p represent the proportion of sites found to have the values of ω indicated. ω = dN/dS. PSS – list of codon positions found to be under selection

Population	Model	p0	p1	p2	ω0	ω1	ω2	Sig	PSS
Ireland	M0				1.648				--
	M3	0.659	0.159	0.182	0	1.845	12.192	< 0.001	38F 44D 71L 101Y 128R 131A 161I 170R 173K 182E
	M1a	0.727	0.273		0.01	1			--
	M2a	0.625	0.189		0	1	9.428	< 0.001	38F 44D 71L 101Y 128R 131A 161I 164L 170R 173K 182E 185L
	M7								--
	M8	0.81	0.19				8.965	< 0.001	
Portugal	M0				1.039				--
	M3	0.703	0.17	0.128	0.04	2.402	7.044	< 0.001	38F 128R 131T 170Q
	M1a	0.709	0.291		0.013	1			--
	M2a	0.628	0.2		0	1	5.693	< 0.001	38F 71Y 128R 131T 161F 170Q 173K
	M7								--
	M8	0.798	0.202				5.604	< 0.001	
Greece	M0				1.282				--
	M3	0.667	0.184	0.149	0.031	1.993	8.096	< 0.001	38F 71Y 101F 128R 131T
	M1a	0.699	0.301		0.013	1			--
	M2a	0.6	0.176		0	1	6.363	< 0.001	38F 41V 71Y 101F 128R 131T 161F 170Q
	M7								--
	M8	0.828	0.172				6.632	< 0.001	

Table 3.12.(cont.) dN/dS results for the DQβ1 exon 2 *locus* on European common dolphins. p represent the proportion of sites found to have the values of ω indicated. ω = dN/dS. PSS – list of codon positions found to be under selection

Population	Model	p0	p1	p2	ω0	ω1	ω2	Sig	PSS
Total	M0				1.23				--
	M3	0.671	0.138	0.191	0.008	1.263	7.492	< 0.001	38F 41V 44N 71Y 101F 128R 131T 161F 164L 170Q 173K 182K
	M1a	0.723	0.277		0.009	1			--
	M2a	0.652	0.193		0	1	6.985	< 0.001	38F 41V 44N 71Y 101F 128R 131T 161F 164L 170Q 173K 182K
	M7								--
	M8	0.807	0.193				7.039	< 0.001	

3.5. DISCUSSION

The analyses of mammalian datasets retrieved from the ORTHOMAM database revealed that most of the candidate genes showed signs of selection. Genes that have shown signs of selection in previous studies have also showed positive results in this analysis, in spite of different species coverage, namely Prestin (Franchini & Elgoyhen, 2006; Li *et al.*, 2010; Liu *et al.*, 2010), TYRP1 (Izagirre *et al.*, 2006; Alonso *et al.,* 2008), SP-C (Foot *et al.*, 2007), and CSN3 (Ward *et al.*, 1997). Such a result is not altogether unexpected given the inclusion of ecologically diverse groups such as monotremates, cetaceans and primates. If a strict criterion is applied for the detection of selection (significant results for both M2a & M8 models), then only 8 out of 14 markers show signs of selection. Model M2a usually assumes higher levels of dN/dS, and as such, only detects stronger cases of positive selection, while M8 constitutes a much less conservative test (Wong *et al.*, 2004). From the cetacean alignments, only one *locus* (LALBA) showed no signs of selection. This result might be related to the very low level of variation found in that marker, as pairwise identity was very high (96.2%, Table 3.4, page 85) and the phylogenetic tree was characterized by very low resolution (Appendix 3.2, page 166). The pattern is similar to that for MC1R, where only the model M3 was significant. Another marker with very low levels of variation was ZP3, but in this case significant signals of selection were detected for both models M2a and M8.

In this study, most markers for which selection was supported only for model M8 showed $\omega 2$ values that were always close to 1 and never higher than 2 (Appendix 3.1, page 144 & 3.2, page 161). This suggests that evidence for positive selection is weak, given that dN/dS values are only marginally higher than what is expected for a neutrally evolving gene. However, dN/dS analysis is based on the relative occurrence of one type of mutation over the other, and therefore power to detect positive selection can be undermined by low variation in the dataset or by saturation of synonymous substitutions when divergence between sequences is high (Wong *et al.*, 2004). It is known that false negatives can occur when using datasets that contain low information (Anisimova *et al.,* 2002; Wong *et al.*, 2004), while false positives are generally thought to be infrequent. In such cases, one mutation will have a disproportionate effect on the dN/dS ratio, even if it has been caused by drift (Anisimova *et al.,* 2001; Wong *et al.*, 2004; Hughes & Friedman, 2005). In addition, when positive selection is only acting on a few branches of the dataset, and purifying selection on the rest, false negatives will also occur, as most of the tree will obscure the signal of the few branches where positive selection is occurring (Yang *et al.*, 2000). As such, a positive result of model M8 might reflect either low

information in the dataset or that selection is only acting in a few branches of the tree analysed.

Evidence for selection is also provided by the comparison of tree topology between markers. The topology obtained in the mammalian trees is generally consistent with previous phylogenies with the exception of the phylogenetic position of the horse (*Equus*), bats (*Myotis*/*Pteropus*), and the hedgehog (*Erinaceus*). The horse position within Perissodactyla is well established, and sister relationship between Perissodactyla and Carnivora is also consistent in the literature (Pumo *et al.*, 1998; Nikaido *et al.*, 2000; Lin & Penny, 2001; Nikaido *et al.*, 2001; Kullberg *et al.*, 2006; Nishihara *et al.*, 2006; Arnason *et al.,* 2008). However, the phylogenetic position of both bats and hedgehogs has long been controversial. Bats are generally accepted as being monophyletic and closely related to Fereuungulata (including the groups Carnivora, Cetarctiodactyla and Perissodactyla) although the exact placement within that group has been inconsistent between studies (Pumo *et al.*, 1998; Nikaido *et al.*, 2000; Lin & Penny, 2001; Nikaido *et al.*, 2001; Nishihara *et al.*, 2006). The hedgehog is probably the most extreme case of inconsistent placement within mammals, having been placed as basal to all eutherian mammals (Nikaido *et al.*, 2001), although it appears to be basal to the Fereuungulata plus Chiroptera (Nishihara *et al.*, 2006; Arnason *et al.,* 2008). The same general inconsistencies are found in this analysis, but more surprisingly, the horse, the hedgehog and the bats will, in some markers, group together in independent clades (horse+bats in CSN3, FGG, HIF1 and SP-C; horse+hedgehog in MYOC; bats+hedgehog in Prestin). Although some of those groupings were supported by low posterior probability and involved markers with low resolution, all these genes were also found to be under positive selection, which suggests that the apparent similarity between these three groups in those markers might reflect shared function. The bottlenose dolphin also groups inconsistently for the NGB gene, and for Prestin when the phylogeny is built on the protein sequence rather than the DNA sequence as in (Li *et al.,* 2010; Liu *et al.*, 2010). When a phylogeny of the protein sequence was built for NGB, the bottlenose dolphin grouped together with primates. Comparing the phylogenetic tree topologies in cetaceans, only the baleen whales showed unexpected grouping for CSN3 (polyphyletic), and sperm whales for ZP3 (discussed below).

The phylogenetic inconsistencies in Prestin were previously attributed to convergent evolution between echolocating bats and cetaceans (Li *et al.,* 2010; Liu *et al.*, 2010). Outer hair cells will change shape in the presence of a mechanical stimuli (Brownell *et al.*, 1985; Ashmore, 1987), thus altering the frequency sensitivity of inner hair cells (Randall *et al.,* 2002;

Santos-Sacchi, 2003). The change in shape of the outer hair cells is controlled by Prestin, a transmembrane protein whose conformation is modified in response to mechanical stimuli from sound waves (Dallos *et al.*, 1991). Knockout of Prestin resulted in significant hearing impairment in mammals (Santos-Sacchi, 2003; Wu *et al.*, 2004) and DNA sequence analysis has shown signs of positive selection in mammals only (Franchini & Elgoyhen, 2006). Furthermore, the results obtained in (Li *et al.,* 2010; Liu *et al.*, 2010) and confirmed here, clearly suggest that this gene is under selection in marine mammals. Therefore, the inconsistent grouping of bats (*Myotis/Pteropus*), the hedgehog (*Erinaceus*) and the horse (*Equus*) found in different markers in this study might reflect a similar phenomenon. In the case of NGB, although no evidence for selection was found in mammals, the inconsistent placement of the bottlenose dolphin suggests that for this group, NGB might have also experienced functional changes, potentially related to the hypoxic conditions of an air breathing mammal in the ocean. Collectively, these results suggest that in animals with highly specialized ecologies, such patterns might be widespread across the genome, and may account in part for the difficulties faced in determining the phylogenetic relationships of such groups with molecular markers, as has been the case with cetaceans (Thewissen, 1998; McGowen *et al.,* 2009).

Although making physiological interpretations for the discrepancies found in Protamine and ZP3 for cetaceans might be tempting, the low resolution found in such markers is a more plausible explanation for such patterns. However, some inferences are possible from comparing both reproduction-related proteins analysed. Many cetacean species are known to be promiscuous (Kenagy & Trombulak, 1986; Hoelzel, 2002), meaning that sperm competition may be high. In this context, it would be expected that female reproduction-related proteins would show less variation than male reproduction-related proteins, as sperm competition would promote the fixation of new protein variants that allow more effective fertilization relative to other males. That is in fact the pattern that can be observed, with Protamine not only having higher variation, but showing good resolution for the cetacean phylogeny, especially when compared to ZP3. Also, the Protamine tree is highly consistent with previously established cetacean phylogenetic trees (McGowen *et al.*, 2009).

The dolphin branch analysis in the mammalian datasets failed to detect positive selection in most markers, even in the markers where more cetacean species were added (with the exceptions of FGG and CAMKA2). Evidence for selection along a particular branch is only expected when the direction or magnitude of selection is very different for that branch

relative to the other branches in the dataset, so failure to detect selection along the dolphin branch might be due to the fact that selection is also present in other branches of mammals as well.

3.5.1. Physiological relevance

Many of the markers where strong evidence for selection were found are known to have well described physiological roles and can be interpreted in an ecological context. Prestin, a marker which has previously been reported to be under selection in mammals with a well described physiological mechanism (Li *et al.,* 2010; Liu *et al.*, 2010) also showed strong evidence for selection in this study (discussed above). MYOC, another marker hypothesized to be interpretable in the context of the increased reliance on sound relative to vision by cetaceans, did show significant support for site models M2a and M8. However, no other evidence were found for its functional relevance as was the case for Prestin, and given that no other cetacean sequences were available and the exact phenotypic effects of this gene are still uncertain, no more interpretations are possible at the moment.

The marker included in this analysis to serve as a negative control (CAMKA2) showed no evidence of selection in the site models. However, it was one of the only two markers to show evidence for selection in the branch analysis of the bottlenose dolphin (*Tursiops truncatus*). Such result is difficult to interpret in light of the fact that the branch analysis gave generally negative results for other markers. The other marker where positive branch results were obtained (FGG; discussed below) had generally good support for selection from the site models as well, while in CAMKA2 most sites had a dN/dS value of 0, suggesting strong purifying selection. These results might reflect that CAMKA2 has a physiologically important role and may have been subjected to positive selection in the cetacean lineage. However, very little is known about the effects that mutations in this gene might have on the phenotype, severely impairing the interpretation of the patterns found in this study.

Milk Proteins

The markers that had stronger support for selection were both milk casein genes (CSN2 and CSN3). These showed support for both M2a and M8 models of selection, in both the mammalian and cetacean datasets. This is further supported by the findings that while most markers screened for the Portuguese coast had very little variation (maximum 3 point mutations of which only one was non-synonymous), the exon 7 of CSN2 exhibited 6 non-synonymous mutations (Table 3.5, page 86). Lactation periods in cetaceans are generally short

but some species can exhibit surprisingly long lactation periods, possibly reflecting different environmental pressures (Hoelzel, 2002). Baleen whales calves must get enough energy from milk to allow them to cope with the long migrations typically done by these animals, and therefore have usually fast lactation periods. On the other extreme, sperm whales (*Physeter macrocephalus*) form stable matrilineal societies where calf protection is increased, and have a correspondingly long lactation time, the longest in cetaceans. These different ecologies and life histories might act as a selective pressures requiring different milk energy content. Caseins are the main constituents of milk proteins, and they form colloidal particles named micelles which are thought to facilitate milk digestion and serve as calcium and phosphate carriers to the infant (Goff, 2010). CSN2 form the interior of the micelle and bind the calcium phosphate, while CSN3 form the surface of the micelle and maintain stability by stopping the other caseins from precipitating in the presence of calcium (Gutierrez-Adan *et al.*, 1996; Fujiwara *et al.,* 1997). Polymorphisms in both these genes are known to change the overall energy characteristics of milk in cattle species (Van Eenennaam, 1991; Manfredi *et al.*, 1993; Martin, 1993; Remeuf, 1993; Amigo *et al.,* 2000; Moioli *et al.*, 2007). Noteworthy in this context is the fact that the shape parameter of the site heterogeneity gamma distribution was always much higher than other markers, indicating that some regions of the sequence are evolving much faster than others. This is consistent with other scenarios typically involving selection on DNA sequences, in which certain parts of the protein have a key role in its physiological function, as for example, in MHC *loci* (Ou *et al.*, 1998). This is also known to occur for CSN3, in which different alleles in a region of the protein encoded by exon 4, designated as casein macropeptide, are known to affect milk protein content and to be under selection in cattle (Ward *et al.*, 1997). Although the present study did not focus particularly on the CMP region, sequences available for cetaceans were from exon 4 (including the CMP region), and have also shown evidence for selection in cetaceans. Taken together, all these results suggest that milk casein genes are fundamental in influencing milk energy content in cetaceans and has been under strong selection throughout their evolutionary lineage.

LALBA also showed strong evidence for selection in the mammalian dataset but not in the cetacean dataset, despite this being the gene with the best representation within this group. LALBA is one of the two sub-units of the enzyme responsible for synthesizing lactose in lactating mammals (Brodbeck *et al.*, 1967). Besides this role, it is also thought to have important antimicrobial and mineral deposition functions (Lonnerdal, 2003), as well as contributing with specific amino acids essential to the infant metabolism (Kelleher *et al.*,

2003). Also, the lack of mammalian tissue regression in the absence of suckling observed in fur seals was related to a mutation causing lack of expression of LALBA (Sharp *et al.*, 2008). Such a mechanism is thought to be an adaptation to the irregular suckling patterns experienced by fur seal pups due to the mother's foraging expeditions (Sharp *et al.*, 2008). Although no evidence was found for positive selection in cetaceans, variation was generally very low and a great majority of sites had a dN/dS value very close to 0. Together with the signals for selection detected in mammals, and the important physiological role known in fur seals (Sharp *et al.*, 2008), the results of this study suggest that this marker might be under strong purifying selection in cetaceans.

Osmoregulation

Genes related to osmoregulation (AQP1, AQP6 and UT-A2) also consistently showed evidence for selection. AQP1 and AQP6 had strong support for both M2a and M8 site models in the mammalian dataset, while UT-A2 had support for M8 model in both mammalian and cetacean dataset. Different lines of evidence indicate that water balance control by means of aquaporins in mammals is dependent on expression regulation, and knockout studies of AQP1 genes resulted in urine concentration deficiencies, especially in situations of prolonged water deprivation (Nielsen *et al.*, 2002). However, studies have also shown that single point mutations might significantly alter the structure of aquaporins leading to changes in permeability function and specificity (Bai *et al.,* 1996; Lagree *et al.*, 1998), with mutations completely changing the protein specificity from water to glycerol (Lagree *et al.*, 1999). Mutations in AQP6 were also shown to alter ion specificity of the molecule (Yasui *et al.*, 1999). Phylogenetic analysis of sequence data from UT-A2 showed that differences in urine concentration between baleen whales and sperm whales were related with differences in UT-A2 gene sequence (Birukawa *et al.*, 2008). This suggests that these genes play an important physiological function and are important in determining adaptation to environments with limited access to drinkable water, such as desert regions, or in the context of cetaceans in a hyperosmotic habitat.

Immunity

All immunity-related markers showed strong signals of selection at all levels analyzed. Both TLR3 and FGG showed evidence of selection in the mammalian dataset, with FGG showing even stronger support when more cetacean sequences were added to the dataset. In addition, FGG was one of the only two markers showing a positive result in the branch analysis of the bottlenose dolphin (*Tursiops truncatus*), suggesting that FGG might be under

particularly strong selection in the cetacean lineage. FGG encodes part of the fibrinogen protein, which is essential to the blood clotting process (Mosesson *et al.*, 2001). Cetaceans have very high levels of parasite load (Evans & Raga, 2001), and in the marine environment, open wounds are a common way for parasite to infect a host (Bush, 2001). As such, given the evidence found in this study, it can be speculated that the marine environment might have imposed a strong selective pressure for more effective clotting mechanism. TLR3 results are consistent with those obtained in the MHC, in the sense that both genes have similar immune functions. However, very few studies have assessed the selective value of this or other toll-like receptors in wild mammals. These results, although preliminary, suggests that immunity genes other than the MHC can exhibit signals of selection in wild animals, thus meeting previous suggestions that research on other immunity related genes can greatly contribute to the understanding of the immune system evolution in wild animals (Acevedo-Whitehouse & Cunningham, 2006), in a manner similar to what studies in the MHC have greatly done in the past.

Diving Adaptations

Evidence for selection was less marked in genes thought to be relevant for the adaptation to constrains imposed by deep diving, although the analysis was impaired by limited representation of cetacean species. SP-C showed support for site model M8 in the mammalian dataset and for models M2a and M8 in cetaceans, suggesting an important role of this gene in adaptation to diving. The composition of the lung surfactant is highly variable among vertebrates. It has a general function of preventing the alveolar walls from sticking to each other during expiration. However, several functions appear to be exclusive to mammals, such as maintaining alveoli stability and reducing the water surface tension in its surface (Daniels & Orgeig, 2001). The results of this study confirm the ones obtained by (Foot *et al.*, 2007), who had previously identified signals of selection in SP-C in marine mammals, and attributed such result to adaptation to high pressures experienced by these animals during diving. However, lung surfactant composition also changes with lower temperatures, and can cause differences in the water surface tension on the alveoli walls, influencing the gas exchange process between air in the alveoli and the blood (Daniels & Orgeig, 2001). Together with the evidence for selection on the mammalian dataset, this suggests that environmental variables other than increased pressure during diving might be acting as selective pressures on this gene. Nevertheless, it appears to be clear that SP-C is under positive selection in cetaceans, and investigating the physiological relevance of these patterns in this group is an exciting field of research.

Genes related with responses to hypoxia showed generally weak evidence for positive selection. Both HIF2 and NGB showed no support for any positive selection sites model, and HIF1 showed support for only the M8 model with dN/dS only slightly higher than 1. This suggests that those markers have an essential physiological role in mammals, and are thus under purifying selection or evolve neutrally. However, no sequences were available for these markers in cetaceans, limiting their interpretation. In particular, NGB was the only marker showing inconsistent placement of the bottlenose dolphin (*Tursiops truncatus*) among the mammalian phylogenetic tree, suggesting convergent evolution (discussed earlier). It is well established that NGB has the effect of protecting brain cells from hypoxia and ischemia, although the exact mechanism by which that is accomplished is still not clear (Sun *et al.*, 2001; Hundahl *et al.*, 2005; Khan *et al.*, 2006; Greenberg *et al.,* 2008). Proposed mechanisms involve more effective transport of O_2, O_2 scavenging under hypoxia conditions, or as a low O_2 sensor triggering other hypoxia related adaptations (Brunori & Vallone, 2007; Greenberg *et al.*, 2008). NGB is also thought to be the main oxygen supplier to the retina (Schmidt *et al.*, 2003). It is thus expected that such gene would exhibit functional changes in cetaceans, and the inconsistent phylogenetic placement suggests this to be true. Therefore, although the results of this study can be considered inconclusive for NGB, they encourage further research on this gene in cetaceans.

Colouration

Colouration genes showed generally little evidence of positive selection. Both MC1R and TYRP1 showed evidence for positive selection in the mammalian dataset, with MC1R having the strongest signal. Both these genes are known to influence skin colouration in mammals, and have been shown to be under selection if different taxonomic groups (Izagirre *et al.*, 2006; Hubbard *et al.,* 2010). However, ASIP is also known to influence colouration and showed no signals of selection. Colouration is controlled by a very complex cascade of reactions involving a large array of different proteins (Slominski *et al.*, 2004). The production of eumelanin by melanocytes is promoted by high levels of MSH protein, which binds to the melanocortin receptor 1 (MC1R). Pheomelanin production is promoted by the ASIP protein, a MC1R antagonist (Slominski *et al.*, 2004). Several mutations are known in these genes that lead to different proportions of black and yellow in several mammalian species (Barsh & Epstein, 1989; Bultman *et al.*, 1994; Kijas *et al.*, 1998; Berryere *et al.*, 2003; Beaumont *et al.*, 2007; Le Pape *et al.*, 2008; Alizadeh *et al.,* 2009; Anderson *et al.*, 2009; Fontanesi *et al.*, 2010), while mutations in the TYRP1 gene are known to cause reduced production of both melanin pigments (Sarangarajan & Boissy, 2001; Alaluf *et al.,* 2003). However, while mutations in the

MC1R and TYRP1 are often due to nucleotide substitutions in coding regions (Hubbard *et al.*, 2010), mutations in ASIP more commonly involve deletions and alternative splicing patterns (Barsh & Epstein, 1989; Bultman *et al.*, 1994; Girardot *et al.*, 2006), making dN/dS analysis of limited use.

In cetaceans, only MC1R sequences were available, and while these showed no evidence for positive selection, dN/dS values were very close to 0, which together with the low levels of variation suggests purifying selection. The general lack of evidence for positive selection in cetaceans does not necessarily suggest lack of adaptive value of colouration in these animals, as several cases are known where differences in colouration are not related to mutations in MC1R (Hubbard *et al.*, 2010). The fact that non-synonymous mutations were found in common dolphin TYRP1 that are consistent with colouration variation found in the wild, indicates that this might be an area for future research.

3.5.2. Limitations of dN/dS methods

Criticisms have been made against the use of dN/dS analysis in unravelling the molecular basis of adaptation outside a few restricted cases, namely the MHC *loci* (Hughes, 2007). One of the strongest points made was that most genes will be under purifying selection in most taxa, and variation will thus be generally low, which undermines the robustness of the test. In addition, most cases where positive selection is found involve levels of dN/dS only slightly higher than 1, making differentiation between positive selection and relaxation of purifying selection difficult (Hughes, 2007). In this study, all the markers where no support for positive selection could be found, did show evidence for purifying selection, as values of dN/dS were very close to 0 (Appendix 3.1, page 144 & 3.2, page 161). Also, in the genes where weak signals of selection were found, dN/dS values were never above 2. However, analysis on a more restricted phylogenetic scale (the order Cetacea in this case) revealed strong signals of selection in markers where only weak signals had been found in the mammalian datasets. Although the patterns found in this study are generally consistent with the limitations highlighted by (Hughes, 2007), this study shows that those limitations do not exclude the possibility that positive selection can be detected in genes related to different physiological systems, particularly if some care is taken regarding the *a priori* hypothesis to be tested. A related criticism made by (Hughes, 2007) was that in most cases where dN/dS analysis has been used to detect selection, a clear biological integration of the functional relevance of the genes analysed was not achieved. However, such integration becomes more feasible as more

information on the molecular basis of different physiological functions becomes available, thus allowing to build sensible *a priori* hypotheses regarding the genes where natural selection is more likely to be detected. When considering all the analyses carried out here, several genes whose physiological function can be integrated within the environmental adaptive pressures the marine environment probably imposed on cetaceans, show evidence of selection using dN/dS analysis.

3.5.3. Variation in European common dolphins

Very little variation could be found in the candidate markers tested for the European common dolphin. Even for those non-synonymous mutations that were detected, their incidence was very low and always found in heterozygous condition, which is consistent with Hardy-Weinberg equilibrium when one allele is rare. Even in Greece, where a bottleneck is expected to have occurred recently (see Chapter 2), no particular changes appear to have occurred in the allele frequency of different candidate genes. These results together with the strong evidence for selection found on a phylogenetic scale suggest that purifying selection is probably acting on these animals. The only exception was the CSN2-255 non synonymous mutation, in which Portuguese locations differentiate significantly from Greece and Ireland with high Fst values (0.13-0.34) but do not differentiate between each other. It is noteworthy that even though very few point mutations were detected in the analysed individuals, CSN2 exhibited 6 non-synonymous point mutations. Even though the mutations screened throughout the entire coast were found to be rare, it does not allow rejecting the hypothesis that these are physiologically relevant, and that they might hold some adaptive value. In fact, caseins are known to have physiologically important functions (see discussion above), and in the phylogenetic analyses they are among the markers exhibiting the strongest evidence for selection. In the mammalian dataset, it is noteworthy that casein genes had the highest levels of variation together with the highest values of the gamma distribution shape parameter, indicating that not only are these genes evolving faster than the other genes analysed, but also in the most heterogeneous fashion along the sequence. dN/dS analysis showed that the highest rate of evolution probably occurs at non-synonymous sites, thus supporting a fundamental role of casein in mammalian ecology. The fact that in CSN2-255 Greece and Ireland do not differentiate is counterintuitive, given that these represent the most dissimilar environments. However, the differentiation is mainly the result of an increase in heterozygote frequency in those locations relative to Portugal, which might reflect a relaxation of the purifying selection

observed in other non-synonymous mutations analysed. However, the fact that these population deviate significantly from Hardy-Weinberg populations suggests local balancing selection. Further investigation the variation of CSN genes in European common dolphin is needed before a more robust conclusion is possible.

Analysis of the variation in the exon 2 of the DQβ1 *locus* showed a different pattern. Levels of variation were high in all locations analysed, and although Fst comparisons were generally low (lower than 0.1), all were significant. The fact that all genetic differentiation statistics showed some correlation with distance by sea between locations suggests that geographic distance might influence the levels of differentiation in the MHC *locus*. This pattern is unexpected given that neither microsatellite data nor any of the other nuclear markers analysed in this study showed any indications of isolation by distance. Although evidence for a reduction in population size can be found in microsatellite data for the Greek population, diversity in the DQβ1 for Greece is not lower than for other European locations. Given the strong evidence for positive selection obtained in the dN/dS analysis, the high diversity found in Greece is likely being maintained through balancing selection, consistent with the findings of other MHC studies on mammals (Bernatchez, 2003). The phylogenetic network is still consistent with a reduction in population size in Greece, as haplotypes found in Greece were generally represented by small number of individuals (often occurring only once) but were scattered among the phylogenetic network. The comparison of the charge profiles distribution of the pocket 4 codons showed that only Greece had a significantly different frequency distribution from the other locations. Although this might suggest that local directional selection for charge profile is operating in the Greek population, the same pattern could have been caused by a reduction in population size.

3.6. CONCLUSION

This study shows that evidence for adaptation can be detected in cetacean physiological functions such as reproduction, osmoregulation, immunity, hypoxia and elevated pressure resistance. All the genes where tests revealed the presence of positive selection, were correlated with physiologically important function in cetaceans, while in genes where this relationship was more tenuous, no such evidence was found (e.g. CAMK2A). Genes under selection were also found to show inconsistent phylogenetic groupings at specific taxa, especially when the phylogenies were based on the protein sequence. This not only supports the idea that such inconsistencies might due to convergent evolution, but also that point

mutations can significantly alter the phenotype in a functionally relevant way. As such, when phylogenetic analysis are carried out using data from nuclear genes underlying physiological functions, interpretation of inconsistent taxonomic grouping would benefit from taking such possibility into consideration. Genes where no evidence of positive selection was found generally showed strong purifying selection, suggesting a more vital function that allowed little change in the protein function. In some cases (such as the MC1R) this finding was at odds with predictions based on physiological relevance. However, such cases involved genes whose effect on phenotype is dependent on complex metabolic pathways, where differences in physiology can be caused by changes in several different genes.

The lack of variation along the European coast suggests that adaptation to different environments found in that region is not promoting any levels of population structuring, but it does suggest purifying selection. The only exceptions are the CSN2-255 non synonymous mutation and the MHC DQβ1 *locus.* However, it's unclear if these patterns relate to any local differences in selective pressures. In CSN2-255, a significant increase in heterozygote frequency in both Greece and Ireland suggest relaxation of purifying selection, while deviations from Hardy-Weinberg equilibrium in both these populations suggests local balancing selection. In DQβ1, Fst comparisons between locations revealed significant differences in allele frequencies, while comparison of the frequency of different pocket 4 charge profiles showed significant differences only for Greece. Although such results suggest location specific differences in selection, the fact that sites identified as being under selection in dN/dS analysis were consistent between locations suggests otherwise, and the evidence for a reduction in population size in the Greek population provides a reasonable alternative explanation for the differences in allele frequencies. A comparison of MHC *locus* variation with regional differences in dolphin parasite load would greatly contribute to answer such questions. In addition, the results obtained for other immune-related genes such as TL3 and FGG provide further immune related genes that can be used to investigate such issues.

Several difficulties were apparent when trying to apply a candidate marker approach to the study of adaptation at a population level. First, many of the markers studied here have very complex splicing patterns, with numerous short exons separated by long introns. Such cases make it very difficult to assess functionally important variation using traditional PCR protocols. Second, using traditional PCR and sequencing protocols to screen a large number of candidate *loci* for a large number of samples is both time and cost consuming, especially without *a priori* knowledge of which markers are likely to be functionally relevant in any

given population. Studies of selection at higher taxonomic levels can be useful in suggesting potential candidates, but as the results of this study have shown, evidence for positive selection at a higher taxonomic level might not reflect positive selection at a population level. As genome scan techniques become more readily available, its application to population level analysis become a more feasible and cost effective strategy. Nevertheless, this study shows that several markers do show signals of selection in cetaceans. At a population level, although only the MHC showed evidence for positive selection, the lack of variation found in other markers suggest purifying selection. In fact, it is interesting to note that none of the markers analysed in this study appeared to evolve in a strictly neutral fashion. Although much of the results produced in this study can be considered preliminary, they strongly suggest that the approach used can undoubtedly contribute to the understanding of the role of adaptation in cetacean evolution.

Chapter 4 – Worldwide Phylogeographic Analysis of the Bottlenose Dolphin (*Tursiops spp.*) Using Mitochondrial Genomes, with Emphasis on the Calculation of Divergence Times

4.1. INTRODUCTION

Bottlenose dolphin (*Tursiops spp.*) is a widespread cosmopolitan species found in all major oceans except polar regions (Folkens *et al.*, 2002). They are characterized by extensive variation in morphology, ecology and behaviour. Group size is generally small but appears to increase depending on behaviour and habitat. Offshore groups are normally larger, as are feeding groups (Shane *et al.*, 1986). Hunting behaviour is also extremely variable and includes trapping schooling fish against the surface (Bearzi *et al.*, 1999), chasing and trapping individual fish against the shoreline (Silber & Fertl, 1995), capturing fish buried in the sand with their rostrums (Sargeant *et al.*, 2007), and using tools as protection from physical damage (Smolker *et al.*, 1997) among other strategies (Sargeant & Mann, 2009). As for social behaviour, a fission-fusion like social structure where males form small stable alliances to herd females, while females form small groups of related individuals is well described in Australia (Connor *et al.,* 1992; Connor *et al.*, 1999; Möller & Beheregaray, 2004). Studies of bottlenose dolphins from the Gulf of Mexico and the Bahamas (Maze-Foley & Würsig, 2002; Krützen *et al.,* 2003; Parsons *et al.*, 2003) suggest this type of social structure might occur outside of Australia, but it is not known how prevalent the Australian model is in other regions. Therefore it seems that bottlenose dolphins are capable of adapting to a variety of different situations, and the exact ecology and behaviour appears to vary with local conditions.

In spite of this considerable phenotypic diversity and several species proposed, only one species was recognised for many years (Shane *et al.*, 1986). Molecular studies have, however, revealed that some morphological variants do represent distinct evolutionary lineages. One well studied case concerns a morphotype occurring in several coastlines along the Indo-Pacific, characterized by a shorter body and longer beak length, with ventral spotting occurring frequently, that had been suggested to represent the distinct species *T. aduncus* (Wang *et al.*, 2000; Wang *et al.*, 2000; Perrin *et al.,* 2008). In China, two sympatric populations of *truncatus* and *aduncus* morphotypes (Wang *et al.*, 2000) were found to show

reciprocal monophyly for the mtDNA control region *locus*, leading the acceptance of *T. aduncus* as a valid species (Wang *et al.*, 1999). In South Africa, however, where the *aduncus* morphotype was first described, an *aduncus* type population was found to separate not only from other *truncatus* type dolphins, but also from the Chinese *aduncus*, suggesting that *T. aduncus* from South Africa might in fact represent a third species (Natoli *et al.*, 2004). Later, studies on Australian bottlenose dolphins revealed that although animals from southeast Australia grouped with the Chinese *aduncus* type (Wang *et al.*, 1999), bottlenose dolphins from south Australia formed yet another independent lineage in mtDNA phylogenies, distinct from both *T. aduncus* and *T. truncatus* (Möller & Beheregaray, 2001; Charlton *et al.,* 2006; Möller *et al.*, 2008). This together with comparatively high genetic distances between these Australian samples and the other *Tursiops* species, led the authors to suggest that these might belong to yet another unnamed species (designated as the South Australian Bottlenose Dolphin – SABD (Charlton *et al.,* 2006; Möller *et al.*, 2008)). However, neither of these studies included samples from the South African *aduncus* described in (Natoli *et al.*, 2004). In the western North Atlantic, coastal and offshore populations of *T. truncatus* known to have distinct morphologies and ecologies (Mead & Potter, 1995), were shown to be genetically differentiated as well (Hoelzel *et al.*, 1998). Later studies have shown that while the offshore ecotype (WNAP) is related to *truncatus* populations found elsewhere in the world, the coastal ecotype (WNAC) consistently forms an independent mtDNA lineage (Natoli *et al.*, 2004; Kingston *et al.,* 2009; Tezanos-Pinto *et al.*, 2009), though the distinction is less clear for nuclear markers (Natoli *et al.*, 2004; Kingston *et al.,* 2009). Furthermore, in Europe, the Black Sea bottlenose dolphin exhibits several morphological differences as compared with other European populations (Birkun, 2002) and has been proposed as a distinct subspecies, *T. truncatus ponticus* (Committee on Taxonomy, 2009). Population genetic analysis revealed that the Black Sea bottlenose dolphin was significantly differentiated from other European bottlenose dolphins, and also revealed that eastern Mediterranean populations were differentiated from other European populations (Natoli *et al.,* 2005; Viaud-Martinez *et al.*, 2008). Presently, the exact taxonomic situation of the bottlenose dolphin (*Tursiops spp.*) remains unresolved. Nevertheless, two species are commonly accepted within the genus: the Indo-Pacific bottlenose dolphin (*T. aduncus*) distributed through coastal areas of east Africa, Asia and northwest Australia; and the common bottlenose dolphin (*T. truncatus*) widespread through all major oceans, with two subspecies, *T. t. ponticus* in the Black Sea and *T. t. truncatus* elsewhere (Folkens *et al.*, 2002; Committee on Taxonomy, 2009).

Questions have also been raised regarding the monophyly of the genus *Tursiops*. Although morphologically all these ecotypes or species are more closely related to each other than to any other cetacean species, several phylogenetic studies have placed the Chinese *aduncus* and the SABD in lineages more closely related to other dolphin species than to *T. truncatus*, making the *Tursiops* genus polyphyletic (LeDuc *et al.*, 1999; Charlton *et al.,* 2006; Nishida *et al.*, 2007; Möller *et al.*, 2008; Kingston *et al.*, 2009; Xiong *et al.*, 2009). However, phylogenies built using several different genes and including a comprehensive species representation of the order Cetacea, give support for the monophyly of the genus *Tursiops* (McGowen *et al.,* 2009; Steeman *et al.*, 2009).

Several studies have described population differentiation on small geographical scales for the bottlenose dolphin (*Tursiops sp.*) (Dowling & Brown, 1993; Krützen *et al.*, 2004; Sellas *et al.*, 2005; Parsons *et al.*, 2006; Bilgmann *et al.,* 2007b; Nichols *et al.*, 2007; Rosel *et al.*, 2009), with a study in the Gulf of California showing a distinction between coastal and offshore populations similar to the one found in the western North Atlantic (Segura *et al.*, 2006). Such complex patterns are unexpected in a species with long-range dispersal abilities (Shane *et al.,* 1986; Wells *et al.*, 1999) in environments that have few obvious geographical barriers to dispersal. Several authors noted that differentiated populations appear to inhabit regions with known differences between oceanographic features (Natoli *et al.*, 2005; Sellas *et al.*, 2005; Bilgmann *et al.,* 2007b), or exhibit differences in prey choice (Dowling & Brown, 1993; Sellas *et al.*, 2005; Segura *et al.*, 2006). A quantitative analysis on the habitat occupation of the western north Atlantic coastal and offshore ecotypes (Hoelzel *et al.*, 1998), showed that depth and distance to shore were robust predictors of each ecotype occurrence (Torres *et al.*, 2003). Several studies have suggested that while adaptation to local environments have contributed to the morphological and ecological differences found between species/ecotypes, the initial separation is more likely to result from specialization on local prey resources maintained by a complex social organization that favours natal philopatry (Wang *et al.*, 1999; Natoli *et al.,* 2005; Parsons *et al.*, 2006). Bottlenose dolphins do exhibit a large variety of regional hunting strategies (Silber & Fertl, 1995; Smolker *et al.*, 1997; Bearzi *et al.,* 1999; Sargeant *et al.*, 2007; Sargeant & Mann, 2009) and genetic studies of social behaviour of bottlenose dolphins in Australia showed that while individual dispersal was evident (especially in males), reproduction appeared to be somewhat confined to the natal area (Krützen *et al.,* 2004; Möller & Beheregaray, 2004). It was suggested that an alliance-based social organization prevents males from dispersing too far from their native areas (Krützen *et al.*, 2004), while females

might benefit from philopatry due to increased familiarity with food resources and kin protection (Möller & Beheregaray, 2004). Similar patterns of philopatry have been described in bottlenose dolphin genetic studies elsewhere (Sellas *et al.*, 2005; Parsons *et al.,* 2006), while photo-identification studies have also suggested a high degree of site fidelity (Durban *et al.*, 2000; Rogers *et al.*, 2004; Baird *et al.,* 2009).

Determining the factors governing diversification in the genus *Tursiops* has been limited by several factors. First, no study to date has carried out a phylogenetic analysis including all of the main diverging groups. Notably, the South African *aduncus* type, the putative subspecies *T. t. ponticus* in the Black Sea, and the Eastern Mediterranean dolphins have not been directly compared. Second, the lack of good estimates for substitution rate or geologic reference points have prevented good estimates of divergence times among populations and putative species (Wang *et al.*, 1999; Viaud-Martinez *et al.,* 2008). Finally, few markers and relatively short sequences have so far been employed.

In this study, the mitochondrial genome was sequenced for individuals representing most of the best described species/ecotypes within the genus *Tursiops*. The differentiation between the Black Sea and the Eastern Mediterranean bottlenose dolphins was used to calibrate the mitogenomic substitution rate in *Tursiops*. The Black Sea is a semi-landlocked basin whose only connection to the adjacent Mediterranean Sea is achieved through the narrow Bosphorous Strait. However, the Bosphorous Strait has only been established between 10,000 and 7,000 years before present (Gökasan *et al.,* 1997; Kerey *et al.*, 2004), since the Black Sea became isolated from surrounding water masses 10 Myrs before present (Nikishin *et al.*, 2003). As such, bottlenose dolphins could have not entered the Black Sea earlier than at the end of the last glacial cycle. The well described geological history of the isolation between the Black Sea and the Mediterranean Sea can therefore be used to estimate a substitution rate for mitochondrial DNA that can then be used to estimate divergence times between the described bottlenose dolphin species/ecotypes. The data obtained for *Tursiops* will be integrated with other available cetacean mitogenome sequences to assess the monophyly of the genus.

4.2. METHODS

4.2.1. Sampling

Samples were obtained from worldwide locations (Figure 4.1, page 111) representative of well described species and/or ecotypes (Table 4.1, page 111). Samples were obtained from stranded and bycaught individuals as well as biopsies from free ranging animals. Western

North Atlantic samples were available from Rus Hoelzel archived at Durham University, European samples were available from Ada Natoli archived at Durham University, Gulf of California samples were provided by Iris Segura at Durham University, and Australian samples were provided by Luciana Möller at Macquarie University.

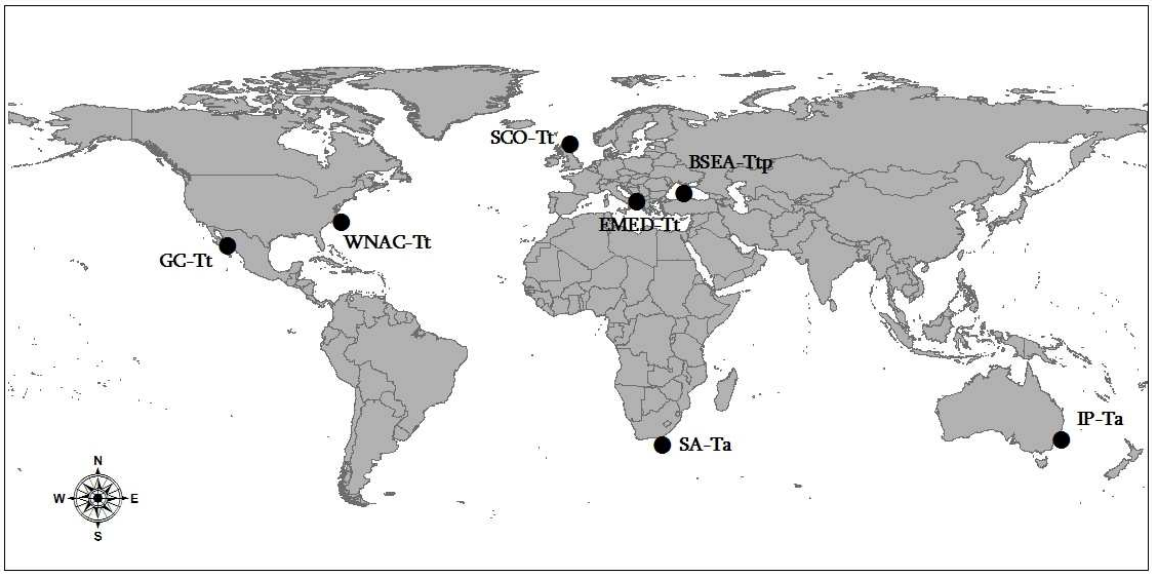


Figure 4.1. Geographic location of the *Tursiops* samples used in this study. Details on the species/ecotypes studied and the number of samples used are described in Table 4.1.

Table 4.1. Number of samples from each *Tursiops* species/ecotypes used in this study. The same code is used throughout this study, and reference corresponds to the publication where the species/ecotype was first described genetically.

Code	Location	Ecotype/Species	N	Reference
WNAC-Tt	Western North Atlantic	Coastal *T. truncatus*	1	(Hoelzel *et al.*, 1998)
SCO-Tt	Scotland	*T. truncatus*	2	(Natoli *et al.*, 2005)
EMED-Tt	Eastern Mediterranean	*T. truncatus*	6	(Natoli *et al.*, 2005)
BSEA-Ttp	Black Sea	*T. truncatus ponticus*	3	(Natoli *et al.*, 2005)
SA-Ta	South Africa	*T. aduncus*	2	(Natoli *et al.*, 2004)
IP-Ta	Australia	Indo-Pacific *T. aduncus*	3	(Möller & Beheregaray, 2001; Wang *et al.*, 1999)
GC-Tt	Gulf of California	Coastal *T. truncatus*	1	(Segura *et al.*, 2006)

4.2.2. Laboratory Procedures

DNA was extracted using a standard phenol:chloroform protocol (Hoelzel, 1998). Whole mitogenome sequences were produced by amplifying two regions with overlapping ends. Primers were designed using PRIMER3 (Rozen & Skaletsky, 2000) algorithm as implemented in the software package GENEIOUS (Drummond *et al.*, 2010), and targeted conserved regions across delphinid species available in GenBank. The first fragment was approximately 8,600 bp

long, including the region between the methionyl-tRNA and ND5 genes. The second fragment was approximately 8,300 bp long, including the region between the ND5 and ND2 genes (Table 4.2, page 113).

Amplification was done using two long range PCR polymerase kits, DynaZyme from Fynnzymes and LA from Takara. Standard PCR profiles for both polymerases are indicated in Tables 4.3 & 4.4 (page 109), although some variation between samples in the number of cycles and $MgCl_2$ concentration was needed for optimal amplification.

PCR products were purified using a PCR Purification Kit from Qiagen following the manufacturer's protocol, and purified DNA was eluted in 1 X TE. Whole genome sequencing was carried out at the Centre for GeoGenetics in the University of Copenhagen by Dr. Thomas Gilbert's research group, using the 454 Life Sciences (Roche) system. Both amplified fragments from each sample were diluted to similar concentrations using a Nanodrop (Thermo Scientific), and subsequently fragmented into shotgun libraries following the manufacturer's indications (454 Life Sciences [Roche]). Libraries from different samples were pooled using Parallel Sequence Tagging (Meyer *et al.*, 2007, 2008). Libraries were tagged with sample specific oligonucleotides containing a *Srf*I restriction site, with untagged sequences removed for sequencing by dephosphorylation (Meyer *et al.*, 2007, 2008). Libraries were then quantified for pooling in equimolar concentrations and digested with *Srf*I restriction enzyme as described in (Meyer *et al.*, 2007). Single stranded DNA was then captured using DNA capture beads and clonally amplified. Beads containing the single stranded DNA were then analysed in a FLX Sequencing System (Roche).

4.2.3. Data Analysis

The obtained output consisted of fragment reads of up to roughly 250 bp. Reads consisting of bottlenose dolphin mtDNA were identified through comparison with a *Tursiops truncatus* mitogenomic reference sequence (GenBank accession number EU557093), and assembled following the method described in (Gilbert *et al.*, 2007). Discrepancies between the 454 sequences and the reference sequence were checked by manual confirmation of the consensus between single stranded reads using the software GENEIOUS (Drummond *et al.*, 2010). All alignments were carried out using the MAUVE (Darling *et al.*, 2010) algorithm as implemented in the software package GENEIOUS (Drummond *et al.*, 2010), and checked manually for inconsistencies. Bottlenose dolphin (*Tursiops spp.*) sequences were then aligned with all available mitogenomic sequences of delphinids, together with sequences from the harbour

Table 4.2. Primers used to amplify the whole mitochondrial genome in two fragments. All primers designed in this study.

Fragment	Primer	Length
Met-ND5	**Met-F1.1:** 5'- GGCCCATACCCCGGAAATGTTGG -3' **ND5-R1.1:** 5'- TGAGTGGAGTAGGGCTGAGACTGG -3'	8598 bp
ND5-ND2	**ND5-F1.1:** 5'- TGATATATGCACTCCGACCCCTAC -3' **ND2-R1.1:** 5'- TCTGTGGCTCGGGGGTTAGG -3'	8339 bp

Table 4.3. Standard PCR mix per tube used for both polymerases used in this study. Some variations were needed for specific samples.

Polymerase	Buffer	MgCl2	dNTP's	Primers	Taq	DNA
DynaZyme	1X	1.5 mM	0.36 mM	0.5 µM	0.5 U	30 ng/µL
LA-Takara	1X	1.5 mM	0.4 mM	0.5 µM	1.5 U	30 ng/µL

Table 4.4. Standard PCR cycling conditions used for both polymerases and fragments amplified in this study. Some variations were needed for specific samples.

Enzyme/fragment	Step	T (°C)	Time	Cycles
DynaZyme: Both fragments	Denaturing	94	1'	1
	Denaturing	94	30"	
	Annealing	65	30"	10
	Extension	68	14'	
	Denaturing	94	30"	
	Annealing	65	30"	15
	Extension	68	14' + 20"/cycle	
LA-Takara: Met-ND5	Denaturing	94	1'	1
	Denaturing	94	30"	
	Annealing/Extension	68	12'	27
	Extension	72	10'	1
LA-Takara: ND5-ND2	Denaturing	94	1'	1
	Denaturing	94	30"	
	Annealing/Extension	68	15'	35
	Extension	72	10'	1

porpoise (*Phocoena phocoena*), sperm whale (*Physeter macrocephalus*), pygmy sperm whale (*Kogia breviceps*), and 3 species of baleen whales as outgroup (GenBank accession numbers in Table 4.5, this page). The *T. aduncus* reference sequence was obtained from a wild dolphin in China, while the *T. truncatus* reference sequence was obtained from a captive animal kept at the Polar and Oceanic Park in the Chinese Shandong Province (Xiong *et al.*, 2009).

Table 4.5. Cetacean species and corresponding GenBank accession numbers used in the phylogenetic analysis.

Species	GenBank Accession Number
Eubalaena australis	AP006473
Balaenoptera physalus	S79330
Eschrichtius robustus	AJ554053
Physeter macrocephalus	AJ277029
Kogia breviceps	AJ554055
Phocoena phocoena	AJ555063
Orcinus orca – resident ecotype	GU187192
Orcinus orca – transient ecotype	GU187173
Globicephala macrorhynchus	HM060333
Globicephala melas	HM060334
Pseudorca crassidens	HM060332
Grampus griseus	EU557095
Lagenorhynchus albirostris	AJ554061
Sousa chinensis	EU557091
Stenella atenuata	EU557096
Stenella coeruleoalba	EU557097
Delphinus capensis	EU557094
Tursiops aduncus	EU557092
Tursiops truncatus	EU557093

Model of sequence evolution was determined using the software TOPALI V2 (Milne *et al.*, 2008), and phylogenetic trees were built using both PHYML (Guindon & Gascuel, 2003) and MRBAYES (Huelsenbeck & Ronquist, 2001) algorithms as implemented in the software package GENEIOUS (Drummond *et al.*, 2010). Branch support in the maximum likelihood tree was assessed through 1,000 bootstrap replicates. The Bayesian tree was built using 2,100,000 replicates and a burn-in length of 210,000 replicates, with a sampling frequency of 400 replicates, run with 4 independent heated chains (preliminary runs revealed this to be appropriate for all chains to reach convergence).

Node ages were estimated using the software BEAST v1.6 (Drummond & Rambaut, 2007). The first tree was generated randomly following a Yule branching model, but tree topology was constrained to keep the several monophyletic groups within the *Tursiops* genus as determined in the phylogenetic analysis. Dolphins were also constrained to be monophyletic, and baleen whales were constrained as outgroup. Two calibration points were used as priors for the calculation of substitution rates and divergence times: the time to the most recent common ancestor (TMRCA) of the lineage including both Eastern Mediterranean and Black Sea dolphins was modelled with a uniform prior bounded between 5kyrs and 10kyrs, according to the published timings for the opening of the Bosphorous Strait (Gökasan *et al.,* 1997; Kerey *et al.*, 2004); The TMRCA of dolphins was modelled with a normal distribution prior with a mean of 10 Myrs and a standard deviation of 1.5 Myrs, based on the observation that delphinid fossils are not found earlier than the late Miocene (Fordyce & Barnes, 1994), and previous divergence dates calculated with molecular data using other independent cetacean fossils as calibration points (McGowen *et al.,* 2009; Steeman *et al.*, 2009; Xiong *et al.*, 2009). Because of the known effect that using recent versus ancient calibration nodes has on the substitution rate (Ho *et al.*, 2005), three independent runs were carried out: 1- both calibration nodes were used; 2- only the delphinids TRMCA based on fossil and molecular data calibration node was used; 3- only the biogeographical node for the separation between Eastern Mediterranean and Black Sea was used. A relaxed uncorrelated molecular clock was assumed, with branch rate distribution following a lognormal distribution (Drummond *et al.*, 2006). The mean substitution rate prior was constrained between 0 and 100 substitutions/site/Myrs, while the standard deviation prior was constrained between 0 and 10. MCMC analysis was run for 40,000,000 iterations with a burn-in length of 4,000,000 iterations and sampling frequency of 4,000 iterations.

4.3. RESULTS

4.3.1. Phylogeographic analysis

Mitogenomic coverage was variable between bottlenose dolphin (*Tursiops spp.*) samples. Regions that were unavailable in at least one sequence were removed from all sequences in the alignment. As such, the resulting alignment included 10,175 base pairs of the mitochondrial genome (Figure 4.2 & 4.3, page 116-117). The alignment contained 3,690 variable sites, of which 2,584 were informative and 107 included gaps., with a transition/transversion bias of 4.081.

Both maximum-likelihood (Figure 4.4, page 119) and Bayesian (Figure 4.5, page 120) algorithms produced well resolved trees with similar topologies and strong support for most

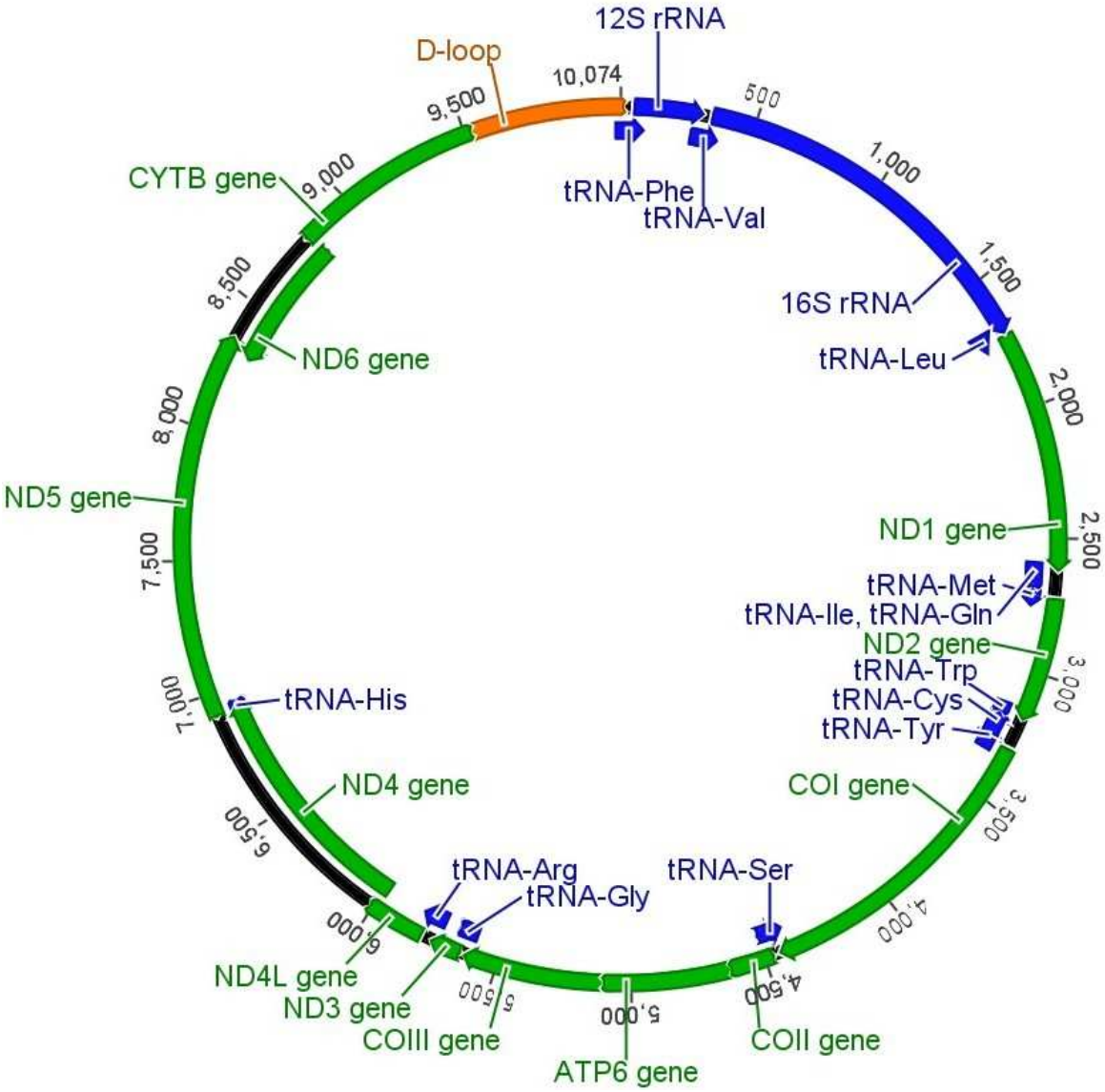


Figure 4.2. Map of the mitogenomic fragment used in the final aligment of this study, based on the *T. truncatus* reference sequence (GenBank accession number EU557093) after all gaps are excluded. Blue rectangles represent RNA genes; green rectangles represent protein genes; orange rectangle represents the hypervariable control region. Image created using GENEIOUS (Drummond *et al.*, 2010).

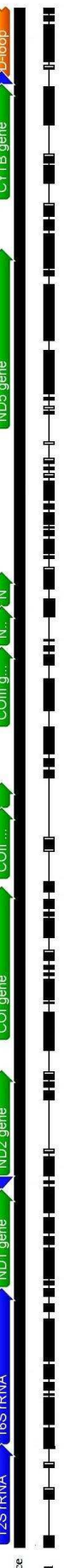


Figure 4.3. Comparison between the *T. truncatus* reference sequence (GenBank accession number EU557093) before and after removing fragments with missing data in the alignment. Black lines between the black rectangles in the clipped sequence represent the fragments removed in the final alignment. Although removed fragments vary in length, they are uniformly scattered through the mtDNA genome. Clipped sequence is represented in Figure 4.2 without gaps. Blue rectangles represent RNA genes; green rectangles represent protein genes; orange rectangle represents the hypervariable control region. Image created using GENEIOUS (Drummond *et al.*, 2010).

nodes. A notable result is that *T. truncatus* and *T. aduncus* are not sister taxa, but *T. aduncus* is more closely related to the common (*Delphinus capensis*) and striped dolphin (*Stenella coeruleoalba*). Most ecotypes analysed within each *Tursiops* species grouped together in well differentiated lineages, except the Eastern Mediterranean dolphins (dark blue in Figure 4.4 & 4.5, pages 119-120) where several haplotypes grouped in the same lineage as other Atlantic ecotypes.

4.3.2. Divergence times between *Tursiops* species/ecotypes

The substitution rates calculated when only the delphinid fossil TMRCA or the Black Sea-East Mediterranean split is constrained differs strongly from each other (Table 4.6, page 121). When both the calibration nodes are used, although the substitution rate is slower than when only the Black Sea-East Mediterranean node is used as calibration, it is disproportionately faster than the rate calculated using only the dolphin fossil calibration. Consequently, divergence times in the latter model are all consistently older than in the other two models. However, the divergence time for the Black Sea dolphins from the eastern Mediterranean based on the slower (fossil calibration) rate is calculated as to 152 thousand years before present (kyrsBP), a date inconsistent with geological history (Gökasan *et al.,* 1997; Kerey *et al.*, 2004). As such, the divergence times calculated using the combined fossil and biogeographical calibration nodes will be used in the rest of this work.

The calculation of divergence times shows that diversification in the genus *Tursiops* appears to be characterized by episodes of diversification in independent lineages occurring in well defined time periods (Figure 4.6, page 122). The divergence of the *T. truncatus* lineage from the one including *T. aduncus*/*S. coeruleoalba*/*D. capensis* is dated to 148.1 kyrsBP, while the divergence of the *T. aduncus* lineage from the *D. capensis* lineage is dated to slightly later at 95.5 kyrsBP. However, the separation between IP-Ta and SA-Ta lineages appears to have occurred roughly simultaneously with the separation of the WNAC-Tt, GC-Tt and European lineages, between 58.7-49.3 kyrsBP. Another period of diversification was dated between 25.1 -20.5 kyrsBP, corresponding to the divergence between the IP-Ta and Chinese *aduncus* (represented by the *T. aduncus* reference sequence) lineages, as well as between the lineage containing the EMED-Tt/BSEA-Ttp ecotypes and the SCO-Tt lineage. This date also corresponds to the separation between the transient and the resident ecotypes of the killer whale (*Orcinus orca*). Diversification within main bottlenose (*Tursiops spp.*) ecotypes broadly coincide with the end of the last glaciation, around 10 kyrsBP, although such dates are

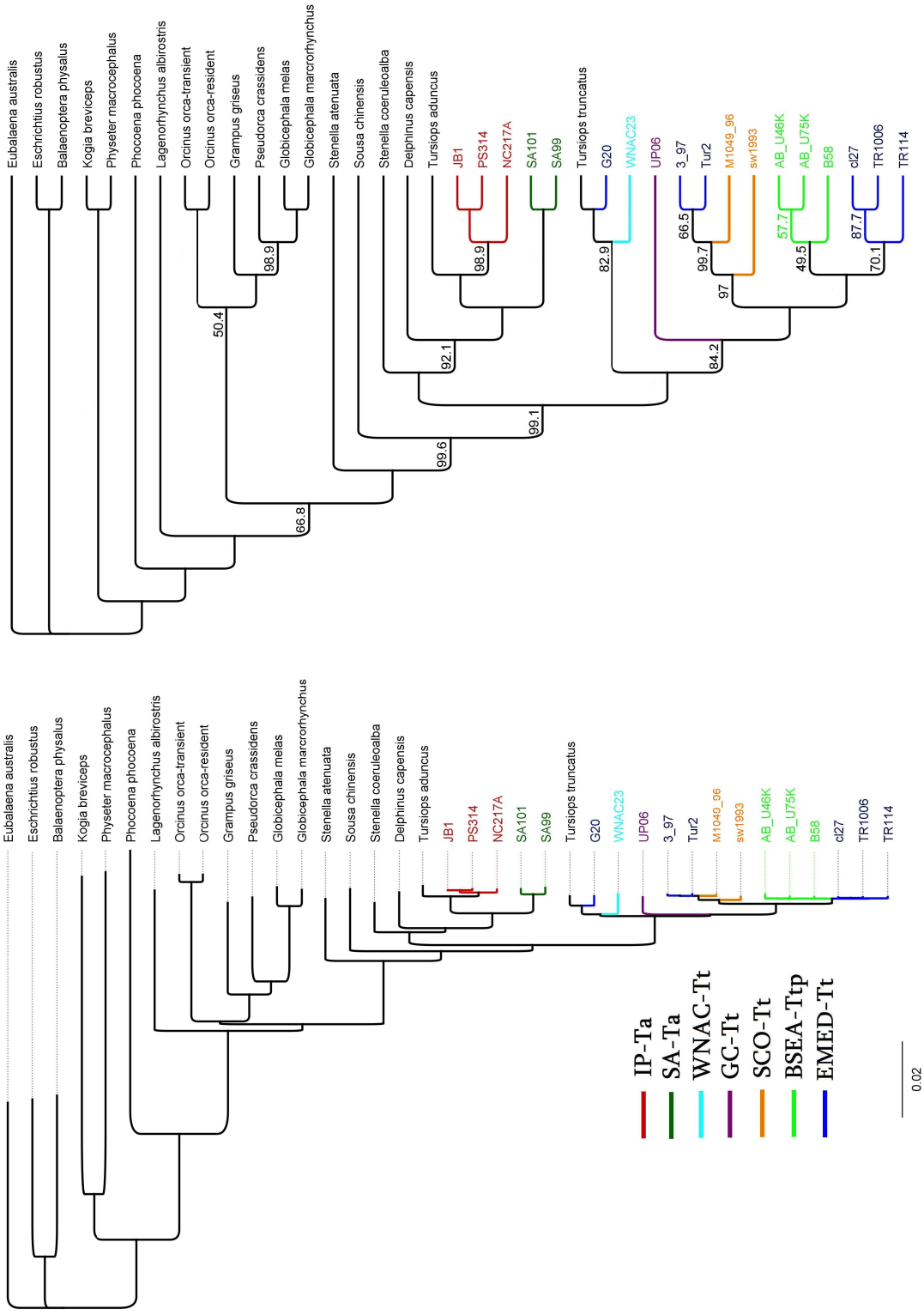


Figure 4.4. Maximum-likelihood phylogenetic tree built using PHYML (Guindon & Gascuel, 2003). Left tree highlights node depth while the tree on the right highlights tree topology, with bootstrap support indicated.

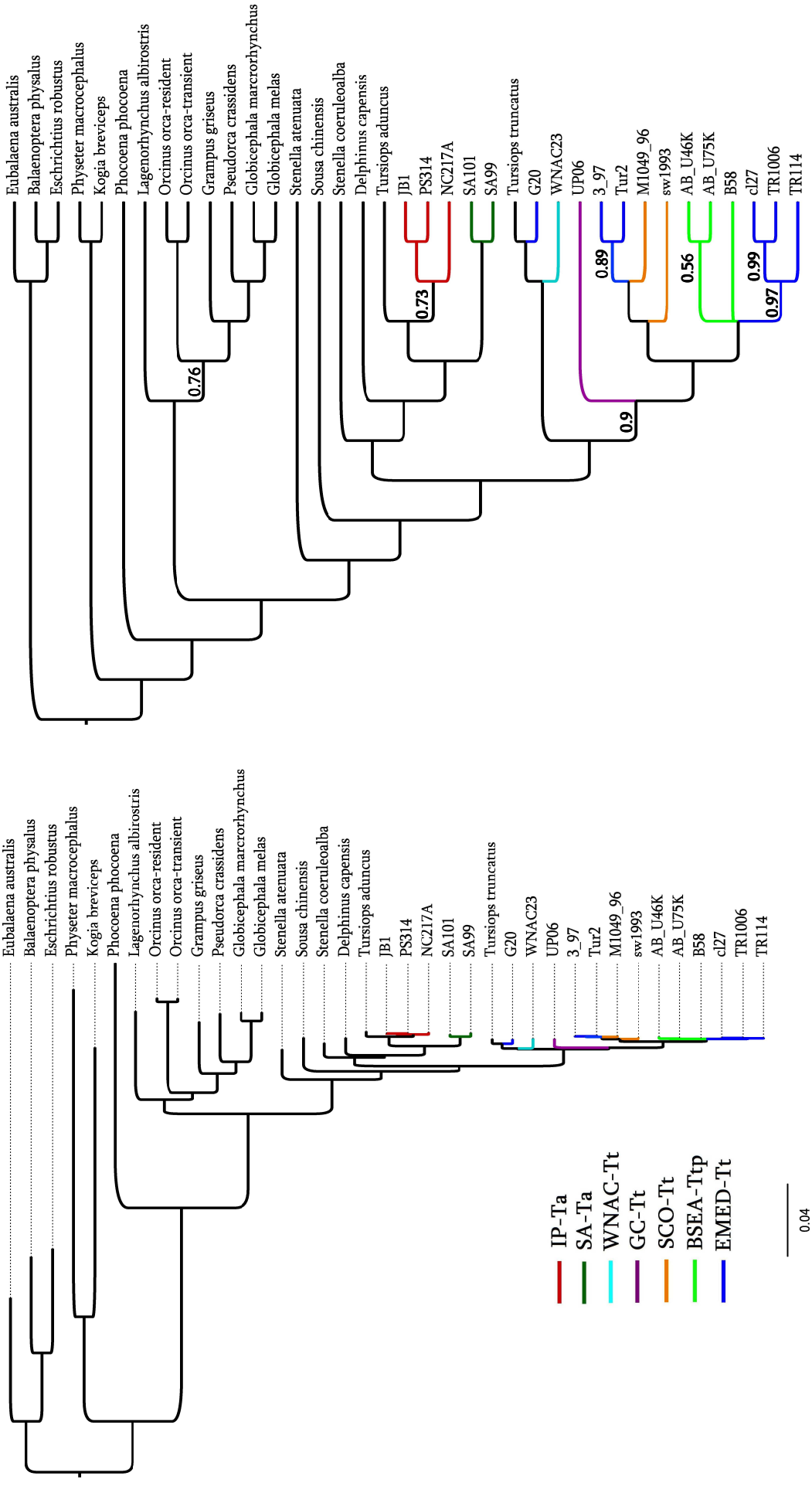


Figure 4.5. Bayesian phylogenetic tree built using MrBayes (Huelsenbeck & Ronquist, 2001). Left tree highlights node depth while the tree on the right highlights tree topology. Posterior probability equals 1 for all nodes, except the ones indicated.

Table 4.6. Mean and 95% confidence intervals (CI) for the several parameters calculated in the BEAST (Drummond & Rambaut, 2007) analysis. 1- both fossil and biogeographical calibration points used; 2- only fossil calibration point used; 3- only the biogeographical calibration point was used. Divergence times represented in one thousand years unit and substitution rates in substitutions/site/Myrs

Parameter	Mean-1	95% CI-1	Mean-2	95% CI-2	Mean-3	95% CI-3
TMRCA: Delphinidae	465	208-775	9245	6002-12168	385	197-630
TMRCA: *T. aduncus*-all	58.68	24.82-98.44	1180	651-1680	49.63	23.27-82.27
TMRCA: *T. truncatus*-all	56.32	24.6-92.93	1129	649-1581	47.27	22.85-76.93
TMRCA: WNAC-Tt/EMED-Tt	51.56	22.51-86.92	1035	598-1476	43.29	21.54-72.42
TMRCA: EMED-Tt/BSEA-Ttp/SCO-Tt	22.93	10.54-38.71	461	254-681	19.38	9.24-31.62
TMRCA: IP-Ta	20.54	8.39-36.2	411	219-632	17.33	7.27-29.85
TMRCA: SA-Ta	14.8	5.24-26.74	295	137-481	12.37	4.77-22.15
TMRCA: SCO-Tt/EMED-Tt	12.64	5.067-22.01	254	117-383	10.67	4.81-18.39
TMRCA: EMED-Tt/BSEA-Ttp	7.208	5.0-9.68	152	78.22-238	6.659	5.0-9.30
TMRCA: BSEA-Ttp	6.966	4.91-9.99	147	72.3-232	6.437	4.52-9.79
TMRCA: EMED-Tt	3.753	1.2-6.7	77.2	26.88-136	3.302	1.00-5.95
Clock rate mean	0.127	0.05-0.20	0.0056	0.0039-0.0084	0.149	0.072-0.229
Clock rate standard deviation	0.256	0.17-0.36	0.255	0.169-0.356	0.259	0.16-0.37

characterized by wide error margins (discussed later). The separation between the Black Sea and the Eastern Mediterranean lineages was dated between 7.1-6.1 kyrsBP, which is consistent with geological data for the opening of the Bosphorous Strait (Gökasan *et al.,* 1997; Kerey *et al.*, 2004). The TMRCA of the delphinids was dated to 464.8 kyrsBP, which is at odds with previous estimates based on molecular data (McGowen *et al.,* 2009; Steeman *et al.*, 2009; Xiong *et al.*, 2009), the significance of which will be discussed later.

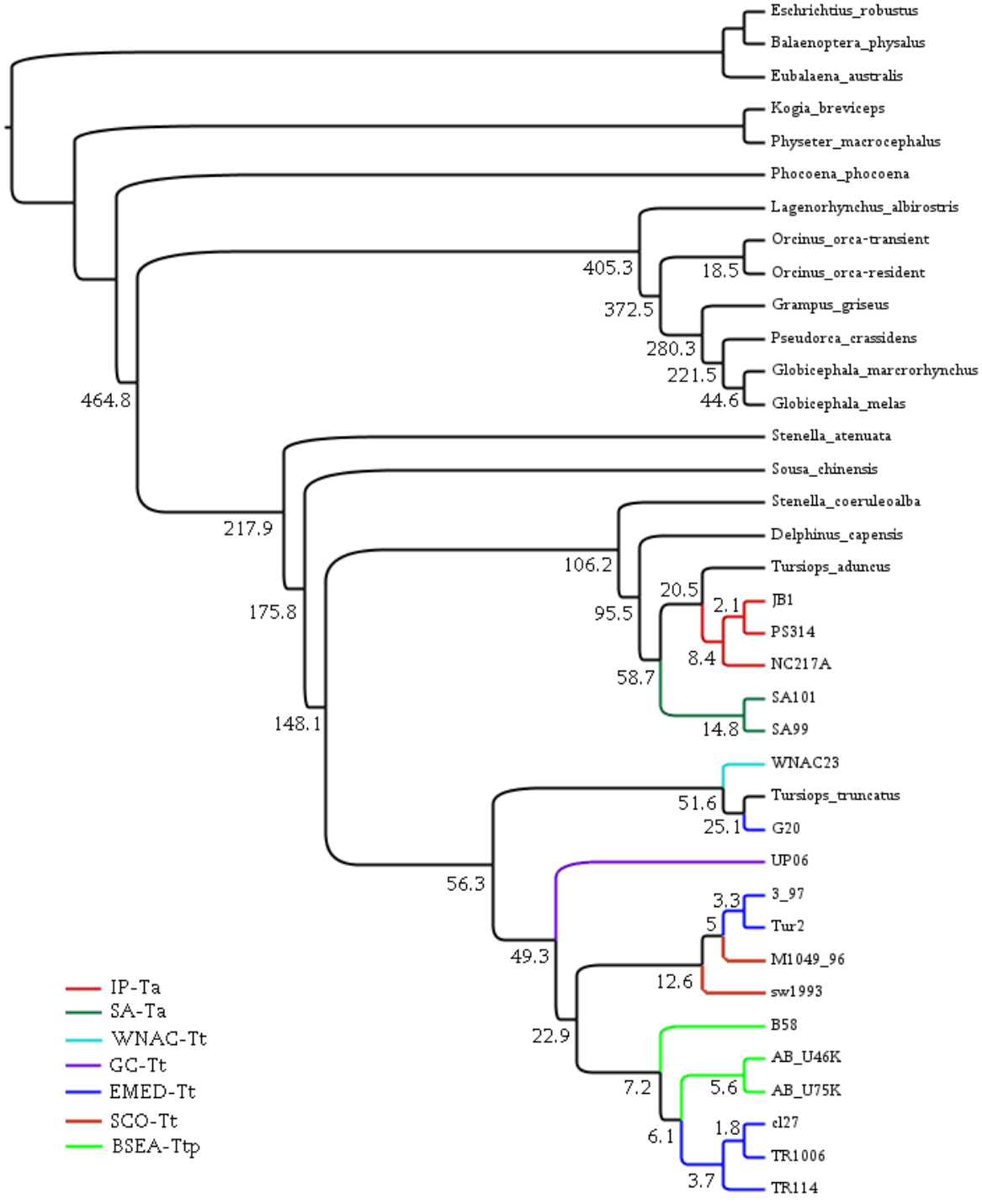


Figure 4.6. Divergence times within cetaceans calculated using BEAST (Drummond & Rambaut, 2007), and both dolphin fossil and biogeographical calibration node (see description of results for details). Branch lengths are not to scale, and only represent the topology. Numbers indicate divergence times in one thousand years unit.

4.4. DISCUSSION

4.4.1. Phylogenetic relationships between the two *Tursiops* species

In this study, the inclusion of a large fragment of the mitochondrial DNA genome has shown to be useful in resolving the relatively shallow nodes that separate different bottlenose (*Tursiops spp.*) species/ecotypes. All nodes were well resolved and had good statistical support. Both currently identified species within the genus *Tursiops* resolved as polyphyletic, with *T. aduncus* being more closely related to both the common (*Delphinus capensis*) and the striped dolphin (*Stenella coeruleoalba*). Although some studies investigating cetacean phylogenetic relationships have found support for the monophyly of *Tursiops* (McGowen *et al.,* 2009; Steeman *et al.*, 2009), most previous studies have not (LeDuc *et al.*, 1999; Charlton *et al.,* 2006; Möller *et al.*, 2008; Kingston *et al.*, 2009; Xiong *et al.*, 2009). However, most of the studies finding support for a polyphyletic *Tursiops* have focused on mtDNA, and given it is a haploid uniparentally inherited and essentially non-recombining molecule, it is unsurprising that the results of this study are consistent with those earlier works. Studies that found support for the monophyly of *Tursiops* have instead integrated information from several different nuclear genes (McGowen *et al.,* 2009; Steeman *et al.*, 2009). However, some studies based on nuclear *loci* were also unable to support monophyly in the genus. A study by Nishida and collaborators (2007) that focused on a sequence of the Y chromosome, also found no support for the monophyly of *Tursiops*, with *T. aduncus* grouping closely with *D. delphis* and *S. longirostris*, while *T. truncatus* grouped with *S. coeruleoalba* in a separate lineage. The study by Kingston and collaborators (2009) that focused on AFLP markers also found no support for the monophyly of the genus, with *T. aduncus* being more closely related to the Fraser's dolphin (*Lagenodelphis hosei*). Given that other studies supporting a monophyletic *Tursiops* (McGowen *et al.,* 2009; Steeman *et al.*, 2009) have included data on several functionally important genes, it is possible that the monophyly of *Tursiops* obtained is the result of molecular convergence, although there is at present no data to support this hypothesis. The phenotypic similarities between both species may thus also represent convergence, even though clear differences are also well described (Wang *et al.,* 1999; Wang *et al.*, 2000). Convergence would suggest an ecological driver in the evolution of these species and local adaptation, although little information regarding the ecology of *T. aduncus* is available.

4.4.2. Dating of divergence nodes

From the dating analysis of the main divergence nodes, it can be seen that two of the calculated divergence times correspond well with interglacial warm periods. The divergence times of both *Tursiops* lineages are broadly consistent with the estimated timing of the Eemian interglacial cycle (roughly between 150-100 kyrsBP), a period of relatively stable climate characterized by warm temperatures (Kukla *et al.,* 1997; NorthGrip, 2004). Also, many divergence times within *Tursiops* ecotype lineages are consistent with the end of the last glacial cycle, around 10 kyrsBP. Other relevant divergence times, however, occur well within the last glacial cycle. The separation of the SA-Ta, the European *truncatus* as well as both *truncatus* coastal ecotypes (WNAC-Tt and GC-Tt) was estimated to around 50 kyrsBP, while the separation of the IP-Ta, and the differentiation between SCO-Tt and EMED-Tt was estimated to around 20 kyrsBP. The last glacial cycle is thought to have been characterized by strong climatic fluctuations, with the extent of the polar ice caps changing considerably both temporally and regionally (Elders & Gibbard, 2004). Although temperatures during the last glacial cycle were generally much lower than temperatures observed during the preceding and following interglacial periods, ice core data reveal a relatively warm period between 60-40 kyrsBP following a particularly cold period (NorthGrip, 2004; Jouzel & Masson-Delmotte, 2010). Independent data suggest that at least in some regions the climate might have been milder during this period. Colonization of southern Australia by humans, for example, appears to have occurred during this period (Bowler *et al.*, 2003) following a local retreat in glacier extension (Barrows *et al.*, 2001). Only the divergence times estimated at around 20 kyrsBP are coincident with a period of extreme glaciations. In fact, the maximum estimated extent of the ice cap during the Last Glacial Maxima (LGM) is thought to have occurred roughly during that period (Yokoyama *et al.*, 2000; Clark *et al.,* 2009). However, the LGM was followed by a fast retreat of the ice caps in the following hundreds of years, which were accompanied by fast changes in the world climate and ocean sea levels (Yokoyama *et al.*, 2000; Weaver *et al.*, 2003; Clark *et al.,* 2004; Clark *et al.*, 2009). The calculation of such dates is, however, faced with several limitations which demand a cautious interpretation, the implications of which will be discussed further below in this section.

Temperature itself is unlikely to have been the driving mechanism in the diversification of these animals. Instead, the dates calculated here suggest that most splits between currently differentiated species/ecotypes occurred in periods of warmer climate relative to the preceding periods. It is thus conceivable that the rapid change between warm and cold cycles

have been accompanied by environmental changes that promoted differentiation in these animals. This could have been caused by adaptation to changing oceanographic conditions or changing patterns of local distribution and abundance of prey resources. In fact, the fast retreat of the ice caps after the LGM would have caused a large runoff of fresh water into the oceans, affecting ocean circulation systems such as the thermohaline current (Weaver *et al.*, 2003; Clark *et al.,* 2004; Clark *et al.*, 2009). Also, changes in the ocean upwelling patterns are known to have occurred shortly after the LGM, giving support to the changing prey resource hypothesis.

A strong influence of changes in ocean productivity on the evolution of cetaceans has been proposed before (Lipps & Mitchell, 1976; Fordyce, 1980; Berger, 2007), and it has been shown that diatom diversity in the oceans seems to be positively correlated with cetacean diversity over geological timescales (Marx & Uhen, 2010). Although studies of present oceanic cetacean diversity distributions found a stronger correlation with sea surface temperature (SST) (Whitehead *et al.,* 2008; Whitehead *et al.*, 2010), SST and diversity at the lower levels of the food chain in the oceans are also generally correlated (Rutherford *et al.*, 1999; Marx & Uhen, 2010). With cetaceans being apex predators, both variables can reflect local productive food webs. The way these climatic changes promoted divergence could have been through adaptation to newly created ecological niches, possibly aided by founder events or population fragmentation resulting from demographic changes. Alternatively, modifications of the general geography of land masses caused by such climatic fluctuations and the accompanying sea level changes could have made previously inaccessible regions available for colonization. For example, during the LGM, most of northern Europe coastlines were inaccessible to cetaceans due to the extension of ice caps. Once these receded, colonization into the newly available coastlines could have led to founder effects as proposed in (Banguera-Hinestroza *et al.*, 2010). Although at present it is not possible to determine the exact mechanisms promoting diversification within *Tursiops*, this study suggests that it occurred simultaneously in different lineages, at specific periods in time which are coincident with fast climatic changes as observed previously in cetaceans (Steeman *et al.*, 2009).

4.4.3. Limitations of the dating analysis

The dates calculated in this study should, however, be taken with caution. Both the DNA substitution rate and the divergence times calculated in this study change drastically depending on whether a fossil or a biogeographical calibration point was used. Such a

phenomenon was first described in a paper by Ho and collaborators (2005) (a pattern called the "lazy-J" (Penny, 2005)), and several cases of it are known in the literature (Ho & Larson, 2006; Genner *et al.*, 2007; Waters *et al.*, 2007; Burridge *et al.,* 2008; Ho & Endicott, 2008; Ho *et al.*, 2008). However, when both the calibration nodes were used in the analysis, the calculated substitution rate was still more similar to the one obtained when only the biogeographical calibration point was used. Several lines of evidence suggest that this rate is more appropriate for the calculation of the divergence times considered here than the one obtained with the fossil calibration point alone. First, according to the fossil calibration, the divergence time between the Black Sea and the East Mediterranean populations occurred around 150 kyrsBP, which is inconsistent with geological data, as the Black Sea was then physically isolated from the Mediterranean Sea (Gökasan *et al.,* 1997; Kerey *et al.*, 2004). Also, the mean substitution rate calculated using both calibration points in this study is 0.127 substitutions/site/Myr, which is similar to previous mtDNA substitution rates calculated from intraspecific variation data in other cetaceans (Ho *et al.*, 2008). The existence of this discrepancy between molecular clock rates calculated using ancient and recent calibration points has been controversial (together with the biological reasons behind it) and is the focus of continuous debate (Woodhams, 2006; Emerson, 2007; Fagundes *et al.*, 2008; Weir & Schluter, 2008; Peterson & Masel, 2009). Namely, such a pattern has been attributed to shallow divergence between analysed sequences and inadequate sampling (Emerson, 2007), uncertainty in the biogeographical calibration nodes used (Fagundes *et al.,* 2008; Weir & Schluter, 2008) and patterns of ancestral population structure and effective population size (Woodhams, 2006; Peterson & Masel, 2009). Uncertainty in the biogeographical calibration point used in this study is unlikely to bias the results obtained, as the bottlenose dolphin could not have entered the Black Sea earlier than 10 kyrsBP. However, it cannot be ruled out that the differentiation between the Black Sea and the eastern Mediterranean lineages did not already exist before the opening of the Bosphorous Strait. Additionally, the branches separating many of the *Tursiops* ecotypes are shallow and sampling is limited to only a few individuals of each ecotype. However, the divergence times for the Black Sea lineage obtained using the fossil calibration is unreasonably high, and for the purpose of this study, the inclusion of a recent biogeographical calibration node thus appears to provide much more reasonable substitution rates and divergence times.

The calculated substitution rate also led to an estimate of the time to the most recent common ancestor (TMRCA) of the Delphinidae at around 500 kyrsBP. Such date is extremely

different from the date of 10 MyrsBP estimated by both fossil data (Fordyce & Barnes, 1994) and previous molecular studies (McGowen *et al.,* 2009; Steeman *et al.*, 2009). The TMRCA of delphinids calculated in this study is likely to be severely underestimated. According to the lazy-J hypothesis, divergence times of over 1 Myrs will be strongly underestimated when using recent biogeographical calibration points (Ho & Larson, 2006), and as such, the substitution rate used is probably inappropriate to calculate the older origin of delphinids (even though a fossil calibration point was included in the calculations).

4.4.4. Taxonomic status within *Tursiops* species

Different ecotypes within both *Tursiops* species have been suggested as independent species (Kingston & Rosel, 2004; Natoli *et al.*, 2004). Most notably, the South African *aduncus* (SA-Ta) has been proposed to represent a different species from the Indo-Pacific *aduncus* (IP-Ta) (Natoli *et al.*, 2004). The results of this study suggest that this is probably accurate, as these lineages appear as reciprocally monophyletic with a calculated divergence time that is older than, for example, the one between the two pilot whale species (*Globicephala sp.*) calculated in this study. However, caution should be taken in interpreting the results of this study in such a way, given the limited sample size used and the single gene tree analysed. No sequences of the recently described South Australian Bottlenose Dolphin (SABD) (Charlton *et al.,* 2006; Möller *et al.*, 2008) were available for this study, and very few samples are available from other locations in the Asian and African coast. Work is currently ongoing to increase sample sizes used and include the SABD to further assess this issue.

Interpretation of the patterns found within *T. truncatus* is more complex. Three different lineages can be defined with divergence times of the same order of magnitude as between both ecotypes of *T. aduncus*: one including the *T. truncatus* reference sequence (from the North Pacific), the western North Atlantic coastal ecotype (WNAC-Tt), and one individual from the Eastern Mediterranean Sea (EMED-Tt); another including the only individual available from the Gulf of California coastal ecotype (GC-Tt); and a third one including all European individuals. However, such lineages are not reciprocally monophyletic and differentiation between them is very shallow, which together with limited sample size, especially within non-European ecotypes such as the western north Atlantic offshore, makes such interpretations speculative at this point. Current work is also focused on increasing samples size and ecotype representation for all *T. truncatus* ecotypes.

4.5. CONCLUSIONS

Although preliminary, the present study shows that the analysis of whole mitochondrial genomes can facilitate the understanding of the phylogenetic relationships within the genus *Tursiops*. The results obtained are consistent with earlier studies (LeDuc *et al.*, 1999; Charlton *et al.,* 2006; Möller *et al.*, 2008; Kingston *et al.*, 2009; Xiong *et al.*, 2009) that together suggest that the genus is not monophyletic. However, multilocus phylogenies suggest otherwise (McGowen *et al.,* 2009; Steeman *et al.*, 2009), and further work is needed. Molecular dating of the divergence times of different species/ecotypes suggests that climatic fluctuations might be promoting differentiation in these animals either by forcing adaptation to a changing environment, both temporally and spatially, by providing new habitats that would promote founder events, or by causing population fragmentation through demographic fluctuations. Limited sampling and coverage of the mtDNA genome are obvious limitations of this study. However, all the nodes were well resolved with high statistical support, something which has been difficult to achieve in previous studies. As such, this study encourages further efforts to achieve full mitogenomic coverage for more representative set of samples. A better coverage of the variation found within the genus *Tursiops*, can further advance our understanding of the evolutionary processes guiding its diversification.

Chapter 5 – Discussion

Speciation is usually initiated when gene flow between subgroups of the ancestral species becomes limited. One of the most widely accepted speciation model considers that such limitation can only occur if an external geographical barrier physically stops migration from occurring (Mayr, 1963; Futuyma, 1998), although models that rely on natural selection as an agent limiting gene flow have long been shown to be feasible (Maynard Smith, 1962; Maynard Smith, 1966; Rice and Hostert, 1993). However, several authors have suggested that an emphasis on geography when classifying models of speciation is misleading and that emphasis should instead be placed on the levels of gene flow observed during divergence (Endler, 1973; Templeton, 1981; Rice & Hostert, 1993; Fitzpatrick *et al.*, 2008; Nosil, 2008). Thus, allopatric speciation models would be at one extreme of divergence without gene flow, while most non-allopatric definitions would fall in the more general model of divergence with gene flow. In such a scenario, the terms allopatric and sympatric become inappropriate because gene flow can be restricted by mechanisms other than geographic barriers. Conversely, natural selection can lead to reproductive isolation independently of a geographic barrier being present (as shown by experimental data compiled in (Rice & Hostert, 1993)).

5.1. Population structure of European common dolphin (*Delphinus delphis*)

In this study, no significant structure could be detected over most of the European distribution of the common dolphin (*Delphinus delphis*) for neutral microsatellite DNA markers, which is the expected pattern for a marine species with high dispersal potential and a fluid social system (promiscuous mating and no stable social bonds). This is also consistent with findings from previous studies, where no genetic differentiation was found in the common dolphin across similar geographic distances, and relatively weak structure found at the oceanic scale in the Atlantic (Natoli *et al.*, 2006; Amaral *et al.,* 2007; Bilgmann *et al.*, 2008; Mirimin *et al.*, 2009). However, the samples collected from the Eastern Mediterranean Sea showed a significant separation from samples collected in the rest of Europe. This is consistent with the findings in (Natoli *et al.*, 2008), who further suggested that such differences reflected differential habitat use, given that other marine species exhibit similar patterns of genetic differentiation in that region (Natoli *et al.*, 2004; Natoli *et al.*, 2005; Perez-Losada *et al.*, 2007; Abaunza *et al.,* 2008). Although similar cases of population structure over small geographic

distances are known for the common dolphin in the Pacific (Rosel *et al.*, 1994; Kingston & Rosel, 2004) and Indian Ocean (Natoli *et al.*, 2006; Bilgmann *et al.,* 2008), the differentiation of Greece is an exceptional occurrence in the North Atlantic (Natoli *et al.*, 2006; Amaral et al., 2007; Mirimin *et al.*, 2009). The results obtained here suggest, however, that genetic drift due to a reduction in effective population size of the Greek population made a significant contribution to the observed pattern of differentiation. This was likely the result of a recent bottleneck, which is consistent with independent data that document a decline in the occurrence of the common dolphin in the Mediterranean Sea over recent decades (Bearzi *et al.,* 2003; Bearzi *et al.*, 2006; Bearzi *et al.*, 2008). The simulation analyses are consistent with these independent demographic data suggesting a very recent timing for the bottleneck (Bearzi *et al.*, 2003). This not only supports the view that such reduction was caused by human activity, but also that such influence can deeply affect the species' genetic composition and observed population structure patterns. Even if conservation measures are applied and the Mediterranean common dolphin recovers to previous abundance levels, it is unclear what the future pattern of population connectivity would be. This highlights the importance of preventive conservation measures, particularly in species with large distribution ranges, as local pressures can lead to strong population fragmentation with potentially lasting effects.

However, selection might be occurring at the molecular level, as the DQβ1 *locus* showed strong differentiation between the eastern Mediterranean Sea and the North Atlantic, both in allele frequencies and the distribution of different charge profiles. Although the extreme demographic event could also explain such differences, several lines of evidence suggest that the patterns found in the DQβ1 may reflect differential selective pressures. First, significant differences in allele frequencies were also found within the North Atlantic between Ireland and Portugal, and there is no signal for a bottleneck in either of these samples. Second, dN/dS analysis strongly suggests that DQβ1 is under balancing selection in all sampled locations, which is expected to reduce the differences between populations. As such, the observed differences are likely to be due to directional selection at the regional scale. Finally, if the Eastern Mediterranean population differentiation in DQβ1 was solely due to drift, it would be expected that some of the other analysed SNPs would exhibit similar patterns, but these other markers show no evidence of structure. The European coast was chosen for its environmental gradient between the Mediterranean Sea and the North Atlantic (see Chapter 2 for details), and as such the differences found in the DQβ1 can potentially be related to regional differences in parasite communities, although such information is currently lacking.

Several studies have reported an excess of non-synonymous mutations at MHC *loci* in cetacean species (Murray & White, 1998; Flores-Ramirez *et al.*, 2004; Baker *et al.,* 2006; Hayashi *et al.*, 2006; Munguia-Vega *et al.*, 2007; Xu *et al.*, 2007; Yang *et al.*, 2008; Nigenda-Morales *et al.*, 2008; Vassilakos *et al.*, 2009; Xu *et al.*, 2009; Xu *et al.*, 2009; Du *et al.*, 2010) consistent with the pattern found in this study, and typical for MHC *loci* across mammals (Hedrick, 1994; Hughes *et al.*, 1994; Horton *et al.*, 1998; Bontrop *et al.*, 1999; Yeager & Hughes, 1999; Aguilar *et al.,* 2004). Some studies that focused on population structure at MHC *loci* also found a coincident structure at neutral markers (Murray *et al.*, 1999; Vassilakos *et al.*, 2009; Du *et al.,* 2010), suggesting differentiation patterns found in the MHC can also be due to drift. The study by Vassilakos and collaborators (2009) showed evidence for local selection in the bottlenose dolphin (*Tursiops truncatus*), although this species also typically shows strong population structure at a regional scale (e.g. Natoli *et al.,* 2004; Tezanos-Pinto *et al.*, 2009). In this study, differentiation at the DQβ1 *locus* can be found between different European locations even though almost no structure could be found at neutral microsatellite *loci* and other functional nuclear genes, which strongly suggests a role of selection.

5.1.1. Analysis of functional diversity

Such inconsistencies in the patterns of differentiation revealed by different markers in the genome is the expected pattern for a system where differentiation is promoted by both selection and drift (Lewontin & Krakauer, 1973; Wakeley, 1996; Wakeley & Hey, 1997). If gene flow is limited solely by an external barrier, then differentiation will mainly occur due to drift and similar differentiation patterns should be observed in all markers. However, if gene flow is limited due to local differences in habitat characteristics or use, relevant functional genes may differentiate first (given that selective pressures are sufficiently strong), while neutral *loci* differentiate over time at a rate dependent on gene flow levels and effective population size (Thibert-Plante & Hendry, 2010; Wu, 2001). Studies assessing the patterns of variation in non-coding *loci* such as microsatellites or anonymous markers such as AFLP's sometimes find evidence for selection by identifying *loci* with outlier patterns of genetic differentiation (Stinchcombe & Hoekstra, 2007; Nosil *et al.,* 2009), sometimes correlated with environmental differences (Nosil *et al.,* 2008; White *et al.*, 2010). However, because no information exists on the functional relevance of these markers (or closely linked markers that could create such patterns through linkage disequilibrium), it's difficult to determine an ecological mechanisms behind such patterns (Hughes, 2007). Additionally, it has been shown

that demographic phenomena such as fast changes in effective population size and complex patterns of population structure can give rise to false positives in such studies (Excoffier *et al.*, 2009).

One of the first well integrated studies comparing levels of divergence between neutral and functional markers focused on closely related species of *Drosophila*, where residuals levels of gene flow after speciation were thought to have reduced the differentiation found in neutral markers (Wakeley, 1996; Wang & Hey, 1996; Wang *et al.*, 1997; Kliman *et al.,* 2000; Noor *et al.*, 2001; Machado *et al.*, 2002). However, many of the genes that were found to be strongly differentiated were known to affect reproductive isolation between the species analysed, and were located in the same chromosome (Wang & Hey, 1996; Noor *et al.,* 2001). Similarly, in African honey bees (*Apis mellifera*) expanding out of Africa, higher Fst values were detected in coding regions relative to non-coding regions, suggesting a generalised effect of selection in populations adapting to new environments (Zayed & Whitfield, 2008). A compelling case-study was reported for species of the genus *Peromyscus* where studies have showed a strong correlation between significant differences in haemoglobin allele frequencies and populations inhabiting different altitudes (Storz *et al.*, 2007). Although a similar level of differentiation was found for mtDNA as well (Gering *et al.*, 2008), different haemoglobin alleles were shown to confer different fitness at different altitudes (Storz, 2007; Storz *et al.,* 2009). Similar patterns have been reported for haemoglobin in other mammalian species (Storz, 2007; Campos *et al.,* 2008).

Although such studies are still rare, they show that focusing on functionally relevant markers in natural populations can provide important insights into the processes promoting population differentiation. Studies of European marine fishes have revealed that functional nuclear markers can exhibit population structure in systems where microsatellite markers failed to do so (Pogson, 2001; Hemmer-Hansen *et al.,* 2007). Little information was, however, available regarding the functional relevance of the markers used in these studies, thus impairing interpretations about which oceanographic features might account for such patterns.

5.1.2. Functional markers that can be useful in the study of adaptation in cetaceans

Selection appears to be detectable in genes controlling for physiologically important functions in cetaceans on several levels. In several markers analysed within the order Cetacea,

non-synonymous mutations are more common than synonymous ones, namely: β-casein, κ-casein, γ-fibrinogen, lung surfactant protein-C, Prestin, Protamine 1 and Zona Pelucida-3. This suggests that, at least for those genes, functional variation has been actively maintained during the course of the group's evolution (Hughes & Nei, 1988; Goldman & Yang, 1994; Muse & Gaut, 1994). In addition, both β-casein and Zona Pelucida-3 resulted in phylogenetic groupings inconsistent with those expected from earlier studies (McGowen *et al.*, 2009), which is a strong indicator of shared function between phylogenetically distinct groups (Li *et al.,* 2008; Li *et al.*, 2010; Liu *et al.*, 2010). These observations are consistent with the prediction that adaptation to the marine environment placed strong constraints on the molecular evolution of physiologically relevant genes. Because of these adaptive constraints, such genes are not expected to follow random patterns of diversification, and will thus not necessarily reflect the species phylogenetic tree. Neutral portions of the genome will diversify due to drift after gene flow has been limited between diversifying groups, and thus will more accurately reflect the species phylogenetic tree. Several previous phylogenetic studies of cetaceans based on functional genes have also resulted in inconsistent groupings (Thewissen, 1998; McGowen *et al.,* 2009).

The genes that showed the strongest evidence for selection were all related to physiological functions that can be speculated to have been more affected during the transition from land to sea, namely osmoregulation, immunity, adaptations to reduced oxygen and increased pressures during diving. Markers that exhibited signals of selection in mammals, however, did not necessarily exhibit the same patterns in cetaceans, and vice-versa. Also, the fact that a particular marker shows strong signals of selection at a phylogenetic level, does not necessarily reflect a meaningful pattern at a population level. Most of the analysed functional markers showed very little variation in European common dolphins suggesting purifying selection, even in cases where several non-synonymous mutations were found, as in β-casein. However, it should be noted that the regions assessed are only fragments of the whole genes, and unless previous knowledge of functionally important regions exist (as was the case of the DQβ1), it is very difficult to know which parts of the gene will be functionally relevant. The analysis of the TYRP1 gene clearly showed this problem, by revealing that from all 8 exons, only 2 had non-synonymous mutations.

Although many of the markers analysed in this study showed no variation in the common dolphin along the European coast, this might only reflect the fact that environmental differences are not strong enough to create such patterns. Cetaceans are ecologically a very

diverse group, and it is unlikely that the same genes will be under selection in all populations/ species. Preliminary studies on higher taxonomic levels can, nevertheless, help to identify good candidate genes to focus in particular case studies. While screening a representative number of genes using a candidate gene approach might be time and cost consuming, the development of large scale genomic screening methods, such as next generation sequencing or SNP microarrays, can allow the evaluation a wider range of candidate markers in a more cost and time effective manner. Nevertheless, several markers have been developed in this study which can potentially be applied to investigate selection in other cetacean species/populations where they might be relevant.

5.1.3. Comparison of the European common dolphin population structure with other cetacean species

The European common dolphin shows a remarkable lack of population structure when compared to other European cetaceans (Garcia-Martinez *et al.*, 1999; Natoli *et al.*, 2004; Natoli *et al.*, 2005; Fontaine *et al.*, 2007; Gaspari *et al.*, 2007; Gaspari *et al.*, 2007; Viaud-Martinez *et al.*, 2008; Banguera-Hinestroza *et al.,* 2010; Fontaine *et al.*, 2010), or other species of delphinids elsewhere, such as the bottlenose dolphin (*Tursiops spp.*) (Hoelzel *et al.*, 1998; Krützen *et al.*, 2004; Sellas *et al.*, 2005; Parsons *et al.*, 2006; Segura *et al.*, 2006; Bilgmann *et al.,* 2007b; Rosel *et al.*, 2009; Tezanos-Pinto *et al.*, 2009; Wiszniewski *et al.*, 2010), the spotted dolphin (*Stenella frontalis*) (Adams & Rosel, 2006), the killer whale (*Orcinus orca*) (Hoelzel *et al.*, 2007), or the false killer whale (*Pseudorca crassidens*) (Chivers *et al.*, 2007). The differentiation found to exist between the Greek and European populations (Natoli *et al.*, 2008), appears to be the result of a recent anthropogenic interference rather than historical limitation of gene flow. Oceanographic features along the European coast that are consistently related to population structure in several marine organisms do not appear to be so in the common dolphin. For example, although several marine species will show population differentiation between the Mediterranean Sea and the North Atlantic (Quesada *et al.*, 1995; Borsa *et al.*, 1997; Chikhi *et al.*, 1997; Naciri *et al.*, 1999; Charrier *et al.*, 2006; Perez-Losada *et al.*, 2007; Abaunza *et al.,* 2008; Comesana *et al.*, 2008; Perez-Portela & Turon, 2008), including cetaceans (Garcia-Martinez *et al.*, 1999; Dalebout *et al.,* 2005; Gaspari *et al.*, 2007; Engelhaupt *et al.*, 2009), such a pattern is not found in the common dolphin. This suggests that the exact patterns of differentiation in cetaceans can be dependent on each species biology and ecology, namely prey choice, mating system, social structure, or other yet unknown mechanisms.

Studies focusing on the feeding ecology and habitat use suggest that the common dolphin tends to occupy areas of high ocean productivity (Brereton *et al.,* 2005; Cañadas *et al.*, 2005; Cañadas & Hammond, 2008), while stomach content studies across Europe showed that it feeds opportunistically on locally abundant schooling fish species with occasional feeding on cephalopods and crustaceans (Young & Cockcroft, 1994; Ohizumi *et al.*, 1998; Silva, 1999; Meynier, 2004; De Pierrepont *et al.,* 2005; Pusineri *et al.*, 2007; Jefferson *et al.*, 2009). This generalist and opportunistic feeding strategy can be a potential mechanism to explain the lack of population structure in Europe when compared to other delphinid species. The common dolphin preference on a prey resource that tends to be patchy but locally abundant, and whose exact distribution likely shifts with time, might promote dispersal rather than site-fidelity thus contributing to the lack of population structure over large distances. However, a mechanism explaining the lack of population structure in the European common dolphin, should also be able to account for the cases where population structure is found on similar geographical scales elsewhere, namely the strong genetic differentiation in common dolphins found in the Pacific (Rosel *et al.*, 1994; Kingston & Rosel, 2004) and in South Africa (Natoli *et al.*, 2006). Both these cases strongly reflect a marked distinction between the short-beaked and the long-beaked morphotypes, a distinction that is not apparent in Europe (Murphy *et al.,* 2006; Westgate, 2007). This variation in skull proportions found worldwide may be correlated with local differences in prey items, a mechanism proposed and generally accepted for other taxonomic groups, namely Darwin finches (Grant & Grant, 1996; Grant & Grant, 2002) and crabs (Yamada & Boulding, 1998). Although differences in prey resources between the short-beaked and the long-beaked common dolphin have yet to be assessed, a positive relationship between dolphin size and prey size has been found in stomach content studies in common dolphins elsewhere (Silva, 1999; Meynier, 2004). Comparatively, the bottlenose dolphin (*Tursiops spp.*) exhibits a high level of genetic differentiation both worldwide and on a regional scale (Dowling & Brown, 1993; Hoelzel *et al.*, 1998; Krützen *et al.*, 2004; Sellas *et al.*, 2005; Parsons *et al.*, 2006; Segura *et al.*, 2006; Bilgmann *et al.,* 2007; Nichols *et al.*, 2007; Rosel *et al.*, 2009). Well differentiated populations in this species will also often show local specialization in prey resources and hunting strategies (Hoelzel, 1998). This is consistent with observations made in *Rhagoletis* flies and other phytophagous insects, in which specialists on different food resources which then mate locally quickly developed reproductive isolation, even in the absence of geographical barriers limiting dispersal (Bush, 1969; Bush, 1994; Linn *et al.*, 2004). Differences in the levels of population structure between oceans observed in the killer whale (*Orcinus orca*) have also been attributed to well described differences in feeding

ecology (Hoelzel *et al.*, 2007; Foote *et al.*, 2009). However, data on feeding habits of both species analysed in this study is still limited, especially for the common dolphin, where most detailed studies have focused on European samples. No definitive statements can be made regarding the role of feeding strategies in accounting for the differences in the level of population structure found in the common dolphin. However, the data collected so far suggests this to be an interesting hypothesis in which to focus further research.

Differences in mating strategies and social structure could also account for such differences. However, much less data is available to allow any conclusions to be made at present. A few well described populations of the bottlenose dolphin exhibit some level of kin association and site-fidelity (Durban *et al.,* 2000; Krützen *et al.*, 2004; Möller & Beheregaray, 2004; Rogers *et al.*, 2004; Sellas *et al.*, 2005), although this pattern is not found everywhere (Defran & Weller, 1999; Defran *et al.*, 1999). Common dolphins in Europe are thought to have a promiscuous mating system (Murphy *et al.,* 2005; Westgate & Read, 2007) and no data at present suggests any level of kin association (Viricel *et al.*, 2008). However, data for the common dolphin is extremely scarce, and essentially absent for many populations worldwide. More data is clearly needed on mating structure, kin association and site-fidelity patterns of different populations in these species, particularly the common dolphin, in order to address this hypothesis.

5.2. BOTTLENOSE DOLPHIN (*TURSIOPS SPP.*) PHYLOGEOGRAPHY

Several authors have suggested that the observed high levels of population differentiation in the bottlenose dolphin (*Tursiops spp.*) might result from specialization in different prey resources, resulting from the environmental changes that occurred at the end of the Pleistocene glaciations (Hoelzel *et al.*, 1998; Wang *et al.*, 1999; Charlton *et al.,* 2006; Natoli *et al.*, 2004; Möller *et al.*, 2008). The results of this study are generally supportive of such a hypothesis. By using a well defined biogeographical calibration point, more appropriate for the calculation of recent divergence times (Ho *et al.*, 2005; Ho & Endicott, 2008), it was estimated that the split between different *Tursiops* species/ecotypes occurred simultaneously in specific time periods, all characterized by fast climatic changes from cold to warmer periods. The lineages leading to both *T. truncatus* and *T. aduncus* split at around the time of the last interglacial period (the Eemian period), almost simultaneously with the divergence of other delphinid species. Although the validity of those two species has generally been accepted, uncertainty regarding the monophyletic status of the genus still existed (LeDuc *et*

al., 1999; Charlton *et al.,* 2006; Nishida *et al.*, 2007; Möller *et al.*, 2008; Kingston *et al.*, 2009; McGowen *et al.*, 2009; Steeman *et al.*, 2009; Xiong *et al.*, 2009). The results of this study support the studies that have found the genus to be polyphyletic, and place *T. aduncus* more closely to the common (*Delphinus sp.*) and the striped dolphin (*Stenella coeruleoalba*) (LeDuc *et al.*, 1999; Charlton *et al.,* 2006; Nishida *et al.*, 2007; Möller *et al.*, 2008; Kingston *et al.*, 2009; Xiong *et al.*, 2009), although the data analysed here represents only a single gene phylogeny.

Changes in the ocean environment caused by climatic cycles has been proposed as a major force in the evolution of cetaceans by several authors (Davies, 1963; Lipps & Mitchell, 1976; Fordyce, 1980; Berger, 2007; Steeman *et al.*, 2009; Marx & Uhen, 2010). However, the exact mechanisms by which such changes have promoted diversification are still a matter of speculation. One hypothesis is that cyclical closing of certain sea basins (such as the Mediterranean Sea) and changes in the availability of coastlines caused by retreating ice caps and the consequent changes in sea levels, promoted diversification through vicariance and drift (Steeman *et al.*, 2009). This is probably a relevant mechanism in the differentiation of coastal ecotypes of the bottlenose dolphin (*T. truncatus*) in which the rise in sea level after the last glaciation could have made new ecological niches available for colonization. It would also account for the observation that many local populations of cetaceans exhibit clear signals of population expansion (Natoli *et al.*, 2004; Amaral *et al.,* 2007; Banguera-Hinestroza *et al.*, 2010). Alternatively, several authors have proposed that changes in the ocean's food web during climatic cycles promoted diversity bursts in cetaceans (Davies, 1963; Lipps & Mitchell, 1976; Fordyce, 1980; Berger, 2007; Marx & Uhen, 2010). Supporting this view are recent studies that have shown a correlation between cetacean diversity and productive food webs, both at present and during geological times (Whitehead *et al.*, 2008; Marx & Uhen, 2010; Whitehead *et al.*, 2010).

In the particular case of the bottlenose dolphin (*Tursiops spp.*), it has been proposed that the Pleistocene glacial cycles provided new habitats that were occupied by small founding populations that then adapted to local environmental conditions (Hoelzel *et al.*, 1998; Wang *et al.*, 1999; Natoli *et al.*, 2004; Charlton *et al.,* 2006; Möller *et al.*, 2007; Möller *et al.*, 2008). Such a process could be promoted by a complex social structure and high levels of site fidelity. Independent studies suggest that the bottlenose dolphin does exhibit some level of site-fidelity (Durban *et al.*, 2000; Krützen *et al.*, 2004; Möller & Beheregaray, 2004; Rogers *et al.*, 2004; Sellas *et al.*, 2005; Parsons *et al.*, 2006; Baird *et al.,* 2009), while stable isotope studies have shown that such habitat fidelity might be related to prey choice (Barros *et al.*, 2010),

especially in the western North Atlantic where dietary segregation between coastal and offshore animals was detected in teeth samples spanning a 100 year period (Walker *et al.*, 1999). This is also supported by studies showing that females occupying distinct habitats will exhibit different hunting strategies (Hastie *et al.*, 2004), and that such behaviours can be transmitted from mother to calf (Smolker *et al.*, 1997; Krützen *et al.,* 2005; Weiss, 2006).

Alternatively, a change in the ocean productivity and upwelling patterns during colder periods (Weaver *et al.*, 2003; Clark *et al.,* 2004; Clark *et al.*, 2009) might have led to fragmentation of previously abundant populations due to demographic crashes promoted by reduction in prey resources. Population expansions that time roughly to the early Holocene (after the last glacial maximum) have been suggested for a number of species (Hoelzel *et al.*, 1998; Tolley *et al.*, 2001; Natoli *et al.*, 2004; Amaral *et al.,* 2007; Banguera-Hinestroza *et al.*, 2010), which could be consistent with this theory. The increased effects of drift in the smaller sub-populations would facilitate the fixation of functional mutations (Wright, 1982) that could turn useful for the adaptation to newly available environments.

5.3. CONCLUDING REMARKS

In this study, the relative effects of drift and selection on the differentiation of cetacean populations were investigated. A combination of local-scale population structure, screening for selection in candidate functional genes at different taxonomic levels, and high resolution phylogeographic analysis of a species known for strong differentiation patterns was carried out. The analysis of candidate functional markers showed that evidence for selection can generally be found in physiologically relevant markers in the cetacean order, thus reflecting the extreme adaptations that cetaceans underwent during the transition from a terrestrial environment to a marine one. However, when analysed on a population level in the European common dolphin, most markers studied exhibited a strong uniformity with little population structure. This suggests that strong purifying selection is acting on these markers. The exception was the MHC DQβ1 *locus*, which showed significant structure in Europe, in particular in comparisons between functional differences in the pocket 4 region between Greek and other European individuals. However, the Greek population shows strong evidence of a rapid bottleneck in recent times, likely leading to the observed differentiation in microsatellite markers due to drift. This indicates that the observed difference at the DQβ1 MHC *locus* may also be the product of drift, but differentiation elsewhere (between Portugal and Ireland, together with other factors; see above) suggests the potential for directional

selection at this *locus* as a response to differences in the local parasite composition (as reported earlier for other delphinid species; Vassilakos *et al.* 2009). Excluding the Greek differentiation (which is likely the result of anthropogenic influence), the European common dolphin exhibits very little population structure both in neutral and functional markers. This pattern, although expected for marine species with high dispersal potential, is at odds with the patterns observed in many dolphin species, documented particularly well in the bottlenose dolphin (*Tursiops spp.*). Mitochondrial DNA results obtained in this study are consistent with previous studies clearly separating two species within the genus (LeDuc *et al.*, 1999; Charlton *et al.,* 2006; Nishida *et al.*, 2007; Möller *et al.*, 2008; Kingston *et al.*, 2009; Xiong *et al.*, 2009), although the monophyly of the genus is still open to debate. Both these species are nevertheless characterized by high levels of differentiation both locally and regionally (Dowling & Brown, 1993; Krützen *et al.*, 2004; Natoli *et al.*, 2005; Sellas *et al.*, 2005; Parsons *et al.*, 2006; Segura *et al.*, 2006; Bilgmann *et al.,* 2007b; Nichols *et al.*, 2007; Viaud-Martinez *et al.*, 2008; Rosel *et al.*, 2009). The divergence times of these species/ecotypes occurred almost simultaneously at key periods in the past, characterized by rapid environmental changes from cold to warmer climate. Such a pattern has been described previously for the diversification of cetaceans, observing that cetacean diversity generally increased in periods of warmer climate (Davies, 1963; Lipps & Mitchell, 1976; Fordyce, 1980 Berger, 2007). Several authors have suggested that changes in the availability of prey resources resulting from such climatic fluctuations might be the mechanism driving diversification in cetaceans (Davies, 1963; Lipps & Mitchell, 1976; Fordyce, 1980; Hoelzel, 1998; Berger, 2007). Such interpretation is consistent with observations that several well differentiated bottlenose dolphin ecotypes are known to exhibit differences in prey choice (Dowling & Brown, 1993; Sellas *et al.*, 2005; Segura *et al.*, 2006) and also exhibit signals of demographic expansion (Natoli *et al.*, 2004) reflecting occupation of newly available habitats. The lower levels of population structure exhibited by the common dolphin comparatively to the bottlenose dolphin can also potentially be explained by differences in feeding ecology. The opportunistic nature of the common dolphin foraging strategies (Young & Cockcroft, 1994; Ohizumi *et al.*, 1998; Silva, 1999; Meynier, 2004; De Pierrepont *et al.,* 2005; Pusineri *et al.*, 2007; Jefferson *et al.*, 2009) may benefit from a more fluid social structure promoting associations between unrelated individuals (Murphy *et al.,* 2005; Westgate & Read, 2007; Viricel *et al.*, 2008), thus promoting higher population connectivity. In contrast, the tendency of the bottlenose dolphin in specializing in specific prey types (Silber & Fertl, 1995; Smolker *et al.*, 1997; Bearzi *et al.,* 1999; Sargeant *et al.*, 2007; Sargeant & Mann, 2009), might benefit from a tighter social

structure promoting kin association (Connor *et al.,* 1992; Connor *et al.*, 1999; Maze-Foley & Würsig, 2002; Krützen *et al.*, 2003; Parsons *et al.*, 2003; Möller & Beheregaray, 2004) and maternal transmission of learned foraging strategies, thus leading to the establishment of regional population differences. However, data on these animals' feeding ecology, social structure and site fidelity is still lacking for many well differentiated populations, particularly so for the common dolphin, and such interpretations must remain speculative. Nevertheless, the comparison of genetic differentiation between cetacean species with different ecologies and social structures can improve the understanding of the mechanisms promoting differentiation in these animals. Directing such comparisons at both neutral and functional genetic variation can further help to incorporate such mechanisms in the context of both drift and selection.

Appendix 2.1 — Simplified DIYABC plots

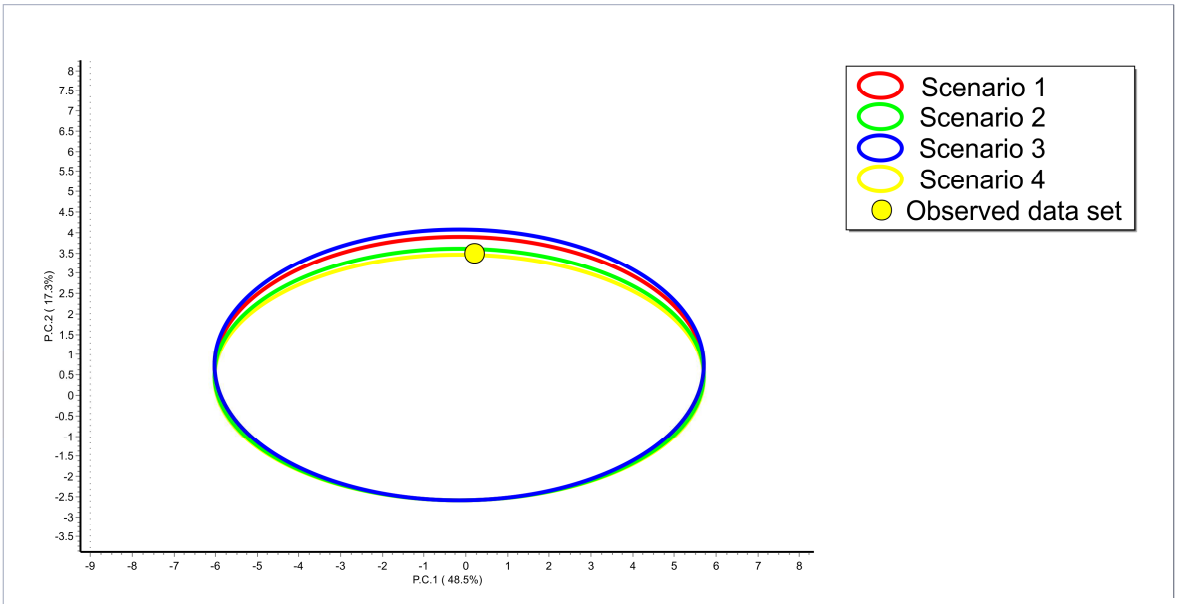


Figure A2.1.1. Simplified PCA plot displaying the fit between scenarios simulated with uniform unconstrained priors and the observed data. Ellipses cover approximately 95% of the data points for each scenario.

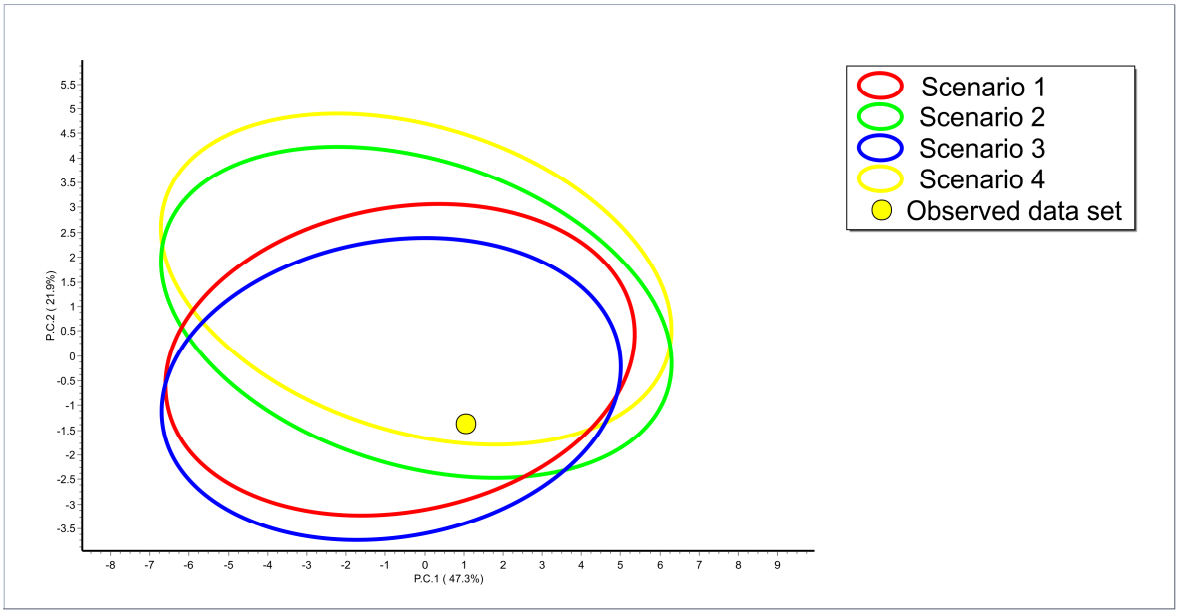
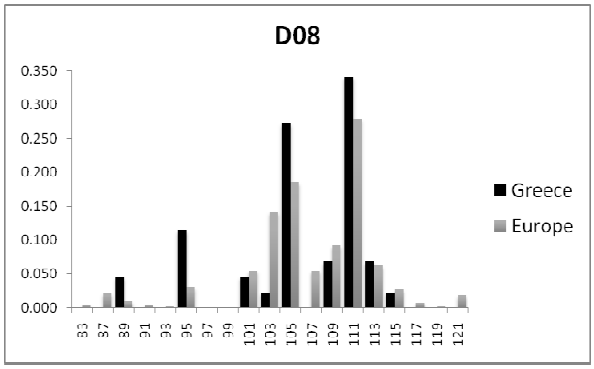
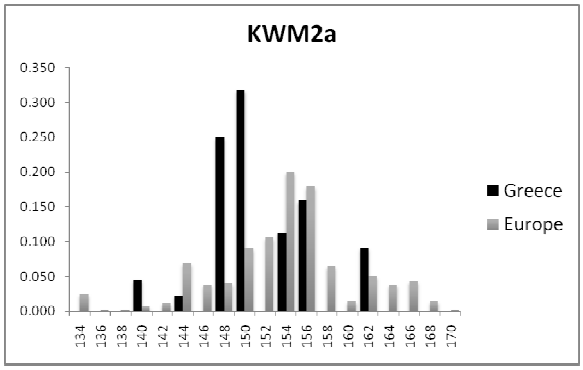
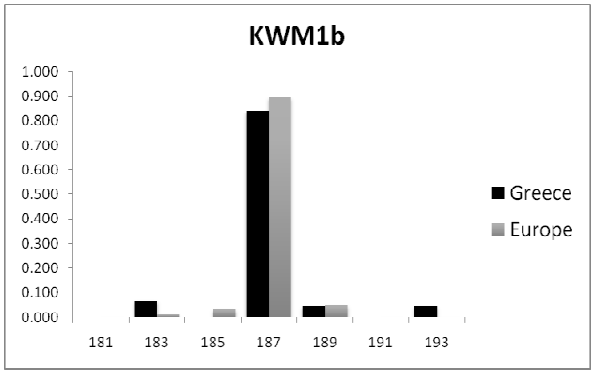


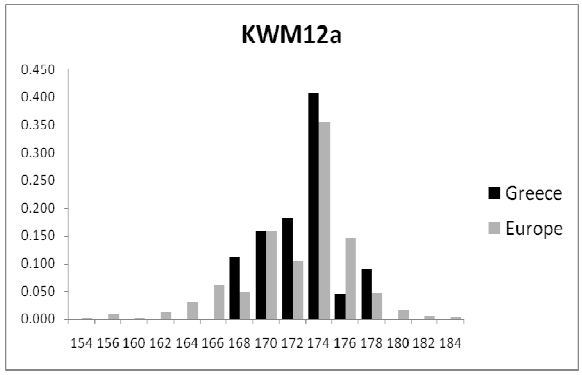
Figure A2.1.2. Simplified PCA plot displaying the fit between scenarios simulated with constrained priors and the observed data. Ellipses cover approximately 95% of the data points for each scenario. Note that the observed data fits the simulated datasets much better than the unconstrained simulations in Figure A2.1.1.

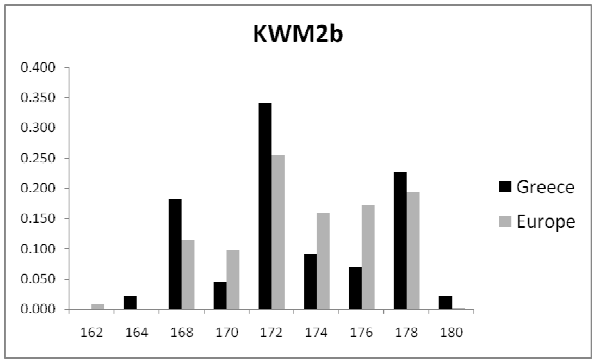
Appendix 2.2 — Allele frequency comparison between European and Greek populations for each microsatellite *loci* genotyped

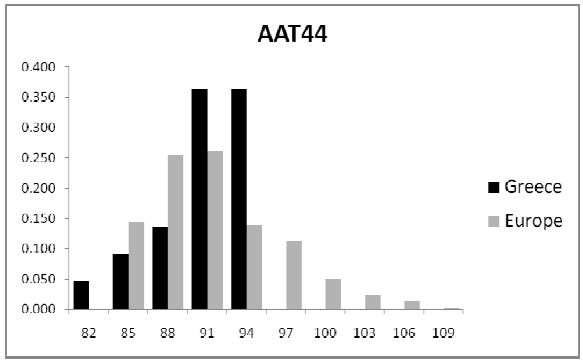


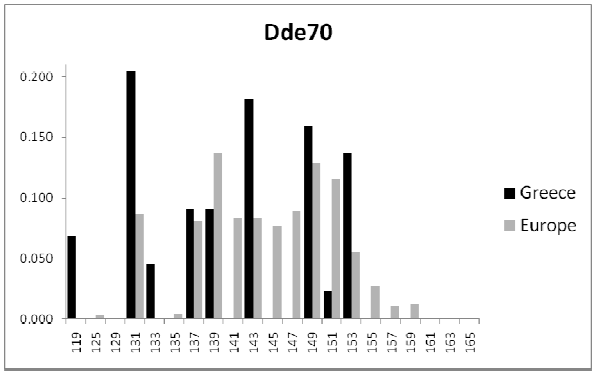


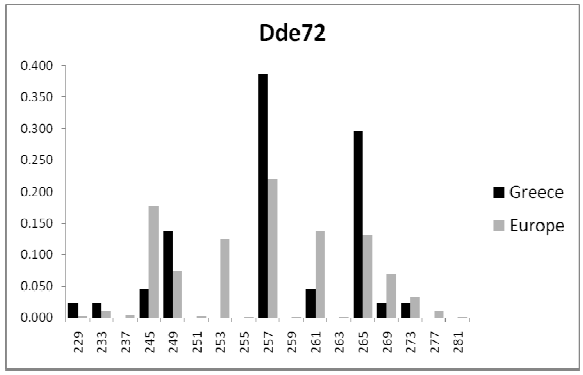


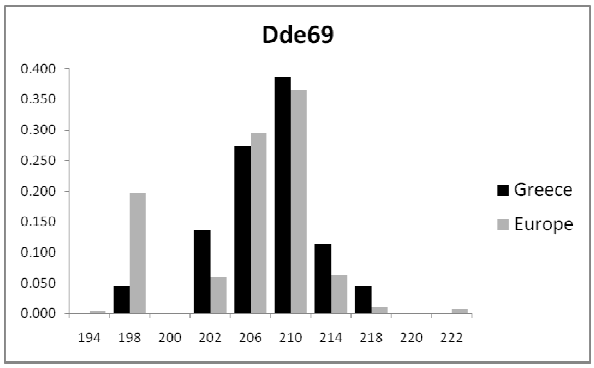








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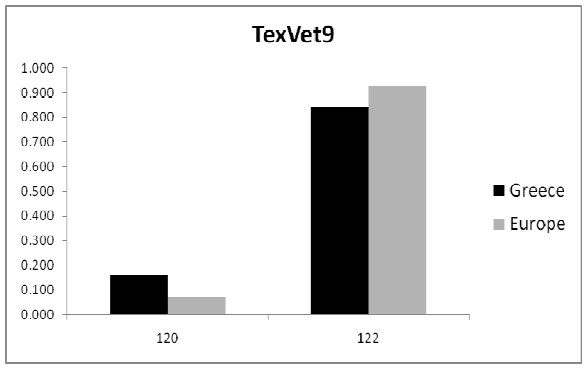
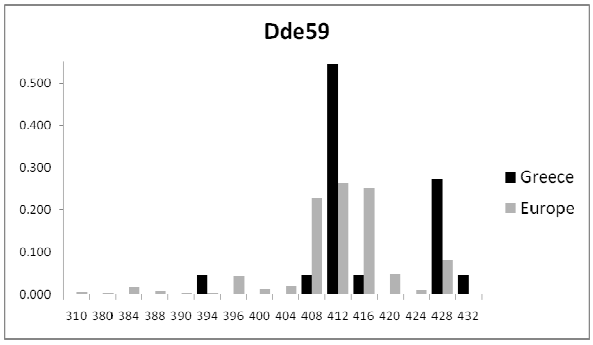
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
194 198 200 202 206 210 214 218 220 222
Greece
Europe


TexVet9
1.000
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
120 122
Greece
Europe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0.100
0.000
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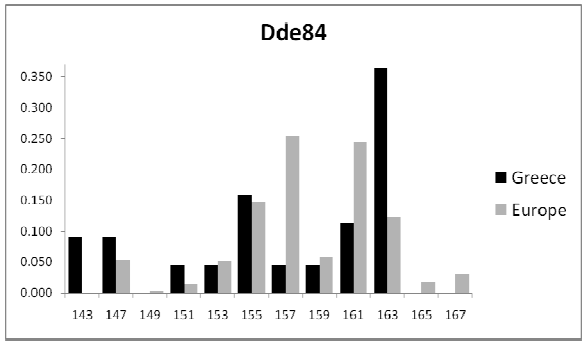
Greece
Europe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0.100
0.050
0.000
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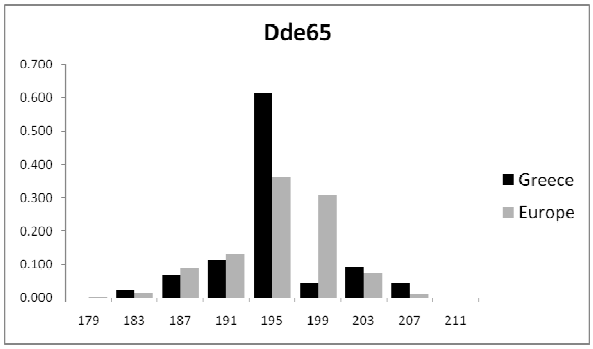
Greece
Europe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0.500
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0.200
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0.000
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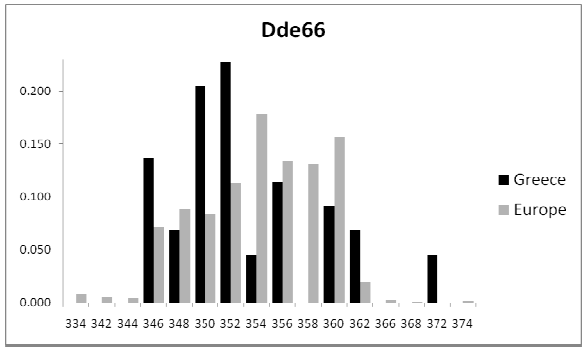
Greece
Europe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0.000
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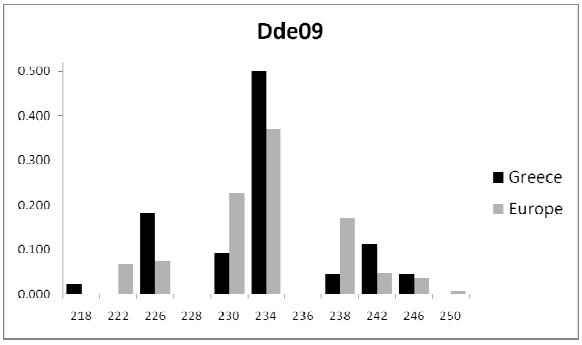
Greece
Europe


Dde09
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0.400
0.300
0.200
0.100
0.000
218 222 226 228 230 234 236 238 242 246 250
Greece
Europe

Appendix 3.1 — Mammalian dataset dN/dS results and candidate markers phylogenetic trees

Table A3.1.1. dN/dS results for the several tested candidate markers in mammals. p represent the proportion of sites found to have the values of ω indicated. ω = dN/dS. PSS – list of codon positions found to be under selection.

Marker	Model	l	p0	p1	p2	ω0	ω1	ω2	p	q	Sig	PSS
CAMKA2	M0	-6854.63				0.018						--
	M3	-6797.27	0.853	0.147	0	0	0.141	25.465			<0.001	
	M1a	-6829.69	0.975	0.025		0.012	1					--
	M2a	-6829.69	0.975	0		0.012	1	26.686			NS	
	M7	-6797.54							0.062	2.204		--
	M8	-6797.54	1	0				1	0.062	2.204	NS	
AQP1	M0	-4070.76	0.786	0.187	0.028	0.006	0.191	1.333				--
	M3	-3899.35	0.914	0.086		0.024	1				< 0.001	107N 368G 398K 626E
	M1a	-3942.13	0.913	0.006		0.024	1	4.227				--
	M2a	-3939.05							0.11	1.036	<0.05	398K
	M7	-3905.11	0.982	0.018				1.999	0.143	2.325		--
	M8	-3894.98									< 0.001	
AQP6	M0	-6372.45	0.46			0.206						--
	M3	-6146.09	0.804	0.4	0.14	0.014	0.231	1.272			< 0.001	14P 17S 23R 29R 38V 53A 131R 140S 164V 362G 407V 413S 623S 749M 773D 779V 800N 803D 809E 812P 815I
	M1a	-6176.87	0.803	0.196		0.088	1					--
	M2a	-6172.36		0.023		0.091	1	2.623			<0.05	17S
	M7	-6161.36	0.91						0.263	0.808		--
	M8	-6146.57		0.09				1.594	0.412	2.19	< 0.001	

Table A3.1.1 (cont.) — dN/dS results for the several tested candidate markers in mammals. p represent the proportion of sites found to have the values of ω indicated. ω = dN/dS. PSS – list of codon positions found to be under selection.

Marker	Model	l	p0	p1	p2	ω0	ω1	ω2	p	q	Sig	PSS
ASIP	M0	-4323.38				0.236						--
	M3	-4251.62	0.571	0.429	0	0.094	0.503	63.517			<0.001	
	M1a	-4266.51	0.755	0.245		0.163	1					--
	M2a	-4266.51	0.755	0.035		0.163	1	1			NS	
	M7	-4240.92							0.737	1.889		--
	M8	-4239.78	0.954	0.046				1.166	0.864	2.637	NS	--
CSN2	M0	-9816.58				1.025						--
	M3	-9723.61	0.285	0.708	0.006	0.328	1.381	6.828			<0.001	458T
	M1a	-9744.4	0.232	0.768		0.228	1					--
	M2a	-9724.72	0.215	0.325		0.26	1	1.798			<0.001	203L 458T 779A 785N
	M7	-9751.73							0.687	0.16		--
	M8	-9724.83	0.522	0.478				1.632	1.072	0.645	<0.001	
CSN3	M0	-7995.28				0.842						--
	M3	-7947.5	0.67	0.33	0	0.596	1.551	47.788			<0.001	
	M1a	-7955.06	0.379	0.621		0.392	1					--
	M2a	-7939.34	0.33	0.105		0.423	1	2.393			<0.001	104S 122W 392E 536P
	M7	-7960.67							1.382	0.429		--
	M8	-7939.78	0.874	0.126				2.207	1.888	0.679	<0.001	

Table A3.1.1 (cont.) — dN/dS results for the several tested candidate markers in mammals. p represent the proportion of sites found to have the values of ω indicated. ω = dN/dS. PSS – list of codon positions found to be under selection.

Marker	Model	l	p0	p1	p2	ω0	ω1	ω2	p	q	Sig	PSS
FGG	M0	-12230.6				0.225						--
	M3	-11752.8	0.657	0.343	0	0.048	0.686	54.956			<0.001	
	M1a	-11775.2	0.696	0.304		0.069	1					--
	M2a	-11772.9	0.693	0.011		0.069	1	2.371			NS	
	M7	-11726.5							0.255	0.703		--
	M8	-11720.9	0.947	0.053				1.392	0.295	1.044	<0.001	
FIT1	M0	-5793.7				0.065						--
	M3	-5710.25	0.881	0.119	0	0.028	0.42	53.82			<0.001	
	M1a	-5725.46	0.937	0.063		0.04	1					--
	M2a	-5725.46	0.937	0		0.04	1	23.899			NS	
	M7	-5708.39							0.236	2.564		--
	M8	-5705.22	0.981	0.019				1	0.304	4.345	<0.001	
FIT2	M0	-6540.99				0.131						--
	M3	-6389.66	0.725	0.275	0	0.04	0.405	22.9			<0.001	
	M1a	-6432.28	0.853	0.147		0.08	1					--
	M2a	-6432.28	0.853	0		0.08	1	24.963			NS	
	M7	-6380.47							0.38	2.163		--
	M8	-6380.47	1	0				1	0.38	2.163	NS	

Table A3.1.1 (cont.) — dN/dS results for the several tested candidate markers in mammals. p represent the proportion of sites found to have the values of ω indicated. ω = dN/dS. PSS – list of codon positions found to be under selection.

Marker	Model	l	p0	p1	p2	ω0	ω1	ω2	p	q	Sig	PSS
HIF1	M0	-14078				0.136						--
	M3	-13836.6	0.716	0.284	0	0.03	0.449	75.855			<0.001	
	M1a	-13879.8	0.874	0.126		0.077	1					--
	M2a	-13879.8	0.874	0		0.077	1	1.067			NS	
	M7	-13820.5							0.272	1.457		--
	M8	-13814.7	0.983	0.017				1.442	0.323	2.043	<0.001	
HIF2	M0	-17990.6				0.111						--
	M3	-17643.5	0.708	0.292	0	0.031	0.358	77.954			<0.001	
	M1a	-17745.5	0.874	0.126		0.071	1					--
	M2a	-17745.5	0.874	0.029		0.071	1	1			NS	
	M7	-17612.6							0.335	2.136		--
	M8	-17611.1	0.984	0.016				1	0.367	2.639	NS	
LALBA	M0	-3948.43				0.504						--
	M3	-3850.36	0.422	0.499	0.079	0.121	0.737	2.621			<0.001	8K 92D 122E 137N 242G 425R
	M1a	-3866.97	0.539	0.461		0.168	1					--
	M2a	-3852.88	0.498	0.057		0.171	1	3.339			<0.001	122E 137N 242G
	M7	-3865.4							0.509	0.551		--
	M8	-3849.54	0.932	0.068				2.762	0.595	0.686	<0.001	

Table A3.1.1 (cont.) — dN/dS results for the several tested candidate markers in mammals. p represent the proportion of sites found to have the values of ω indicated. ω = dN/dS. PSS – list of codon positions found to be under selection.

Marker	Model	l	p0	p1	p2	ω0	ω1	ω2	p	q	Sig	PSS
MYOC	M0	-14244.5				0.175						--
	M3	-13782.3	0.516	0.418	0.067	0.034	0.312	1.259			<0.001	41K 173V 326T 434A 494E 584A 608R 695S 725Q 749A 866R 944S 1013H 1178L
	M1a	-13894.7	0.781	0.219		0.1	1					--
	M2a	-13887.3	0.778	0.008		0.102	1	3.726			<0.001	749A
	M7	-13790.4							0.44	1.497		--
	M8	-13769.5	0.968	0.032				1.846	0.533	2.278	<0.001	
TLR3	M0	-24032.8				0.247						--
	M3	-23349.7	0.655	0.345	0	0.06	0.666	28.013			<0.001	
	M1a	-23388.4	0.734	0.266		0.095	1					--
	M2a	-23382.3	0.73	0.008		0.096	1	2.646			<0.01	
	M7	-23295							0.314	0.862		--
	M8	-23278.2	0.957	0.043				1.5	0.376	1.289	<0.001	
TYRP1	M0	-12960.5				0.152						--
	M3	-12579.5	0.747	0.253	0	0.04	0.568	27.854			<0.001	
	M1a	-12609.7	0.836	0.164		0.07	1					--
	M2a	-12607.8	0.835	0.005		0.07	1	2.424			NS	
	M7	-12547.1							0.239	1.053		--
	M8	-12532.1	0.953	0.047				1.356	0.328	2.169	<0.001	

Table A3.1.1 (cont.) — dN/dS results for the several tested candidate markers in mammals. p represent the proportion of sites found to have the values of ω indicated. ω = dN/dS. PSS – list of codon positions found to be under selection.

Marker	Model	l	p0	p1	p2	ω0	ω1	ω2	p	q	Sig	PSS
UT-A2	M0	-20791.3				0.14						--
	M3	-20300.9	0.724	0.276	0	0.042					<0.001	
	M1a	-20383.7	0.841	0.159		0.078	1					--
	M2a	-20383.7	0.841	0.078		0.078	1	1			NS	
	M7	-20274.6							0.325	1.527		--
	M8	-20262.9	0.942	0.058				1.025	0.44	3.062	<0.001	
NGB	M0	-2832.51				0.086						--
	M3	-2806.93	0.646	0.354	0	0.025	0.213	28.85			<0.001	
	M1a	-2824.24	0.981	0.019		0.08	1					--
	M2a	-2824.24	0.981	0		0.08	1	14.886			NS	
	M7	-2807.3							0.66	6.29		--
	M8	-2807.16	0.996	0.004				1	0.703	6.958	NS	
Prestin	M0	-14965.9				0.097						--
	M3	-14641.6	0.625	0.337	0.038	0.01	0.195	1.163			<0.001	116S 977K 1757V 1787K 1847V 1850H 1883G 1913Q 1919G 1949N 1997V 2147R 2153K 2177K 2255L 2261P 2279T
	M1a	-14714.5	0.918	0.082		0.057	1					--
	M2a	-14710.2	0.918	0.004		0.057	1	4.062			<0.05	1847V
	M7	-14663.8							0.236	1.677		--
	M8	-14640.7	0.977	0.023				1.494	0.324	3.274	<0.001	

Table A3.1.1 (cont.) — dN/dS results for the several tested candidate markers in mammals. p represent the proportion of sites found to have the values of ω indicated. ω = dN/dS. PSS – list of codon positions found to be under selection.

Marker	Model	l	p0	p1	p2	ω0	ω1	ω2	p	q	Sig	PSS
SP-C	M0	-5264.61				0.201						--
	M3	-5137.45	0.561	0.439	0	0.041	0.471	39.309			<0.001	
	M1a	-5146.38	0.84	0.16		0.12	1					--
	M2a	-5146.38	0.84	0.073		0.12	1	1			NS	
	M7	-5117.23							0.392	1.206		--
	M8	-5108.41	0.912	0.088				1.342	0.625	3.217	<0.001	

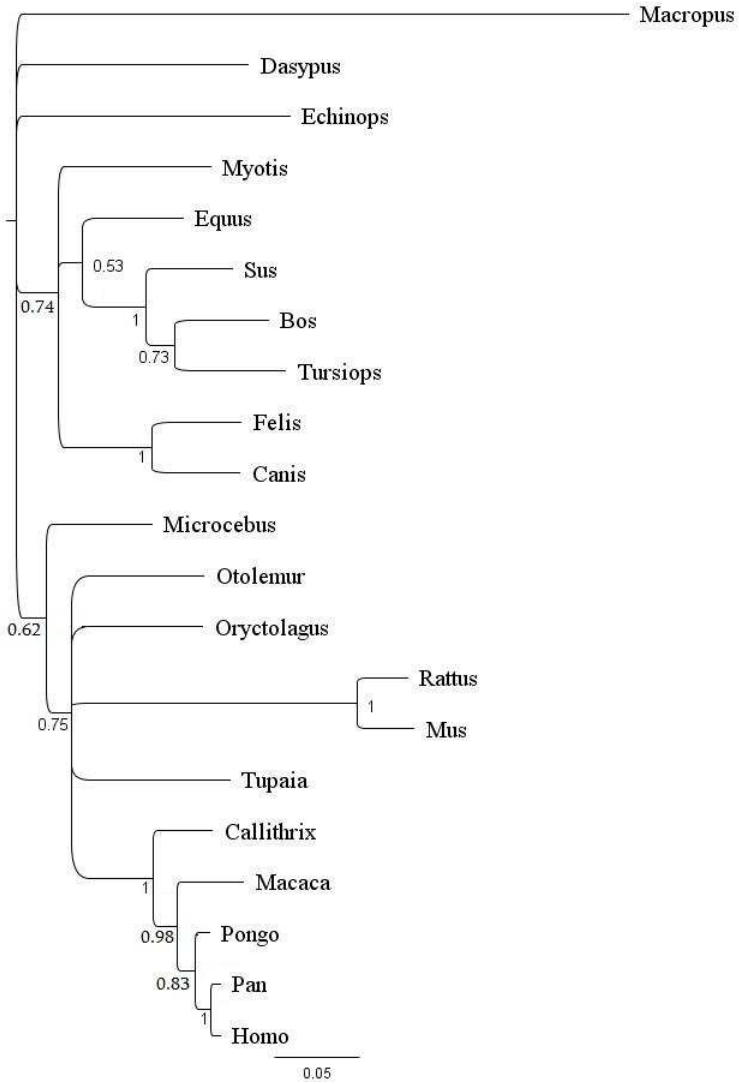


Figure A3.1.1. AQP1 Bayesian tree built for the mammalian dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

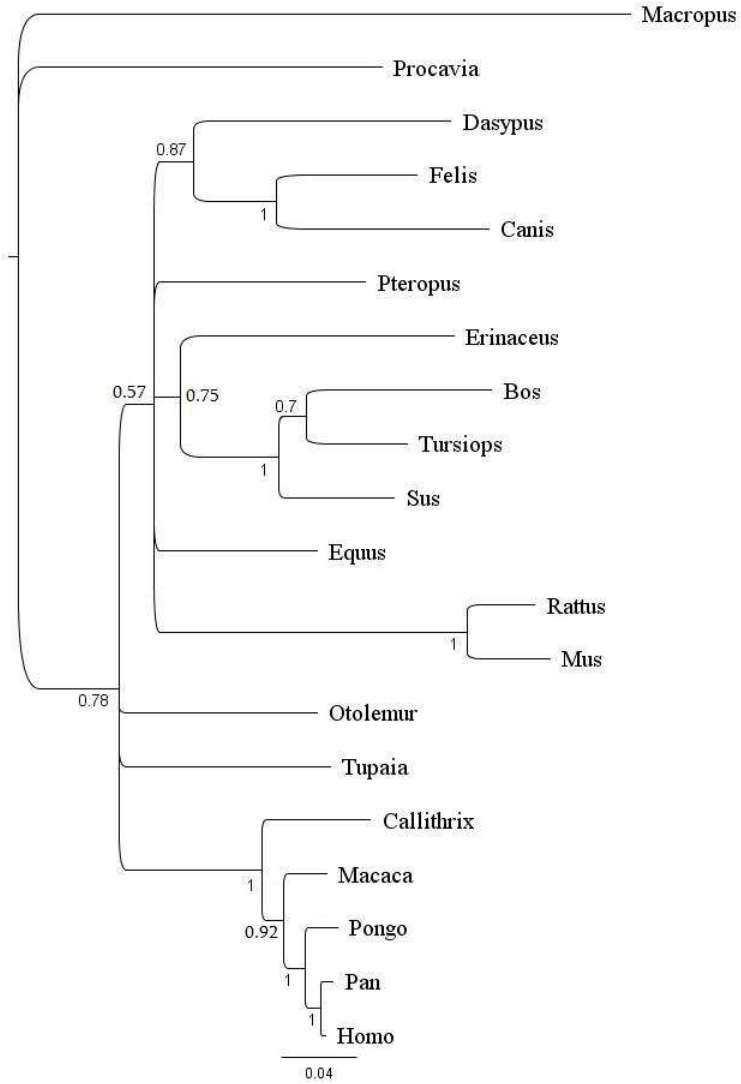


Figure A3.1.2. AQP6 Bayesian tree built for the mammalian dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

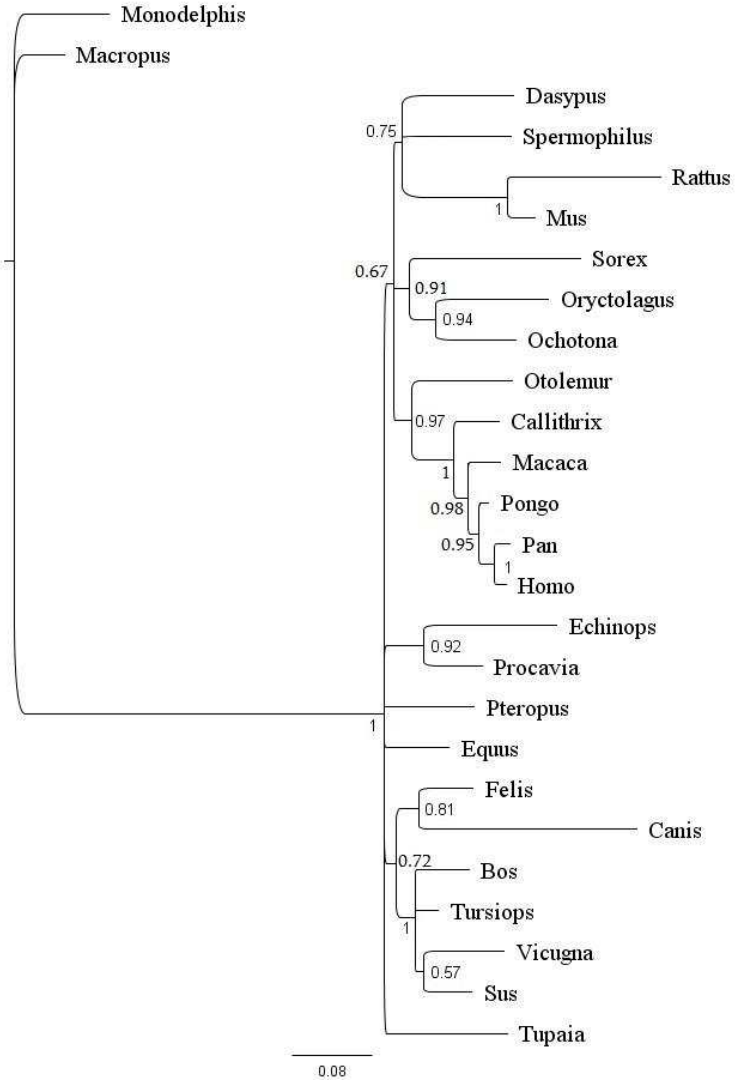


Figure A3.1.3. ASIP Bayesian tree built for the mammalian dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

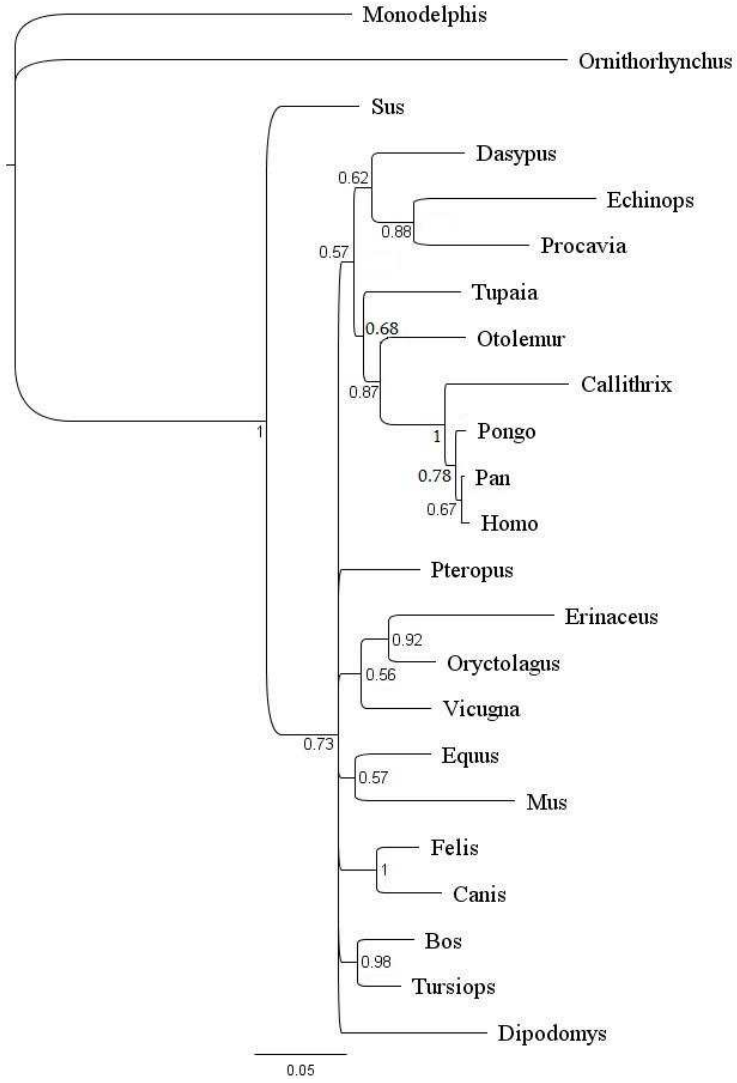


Figure A3.1.4. CAMKA2 Bayesian tree built for the mammalian dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

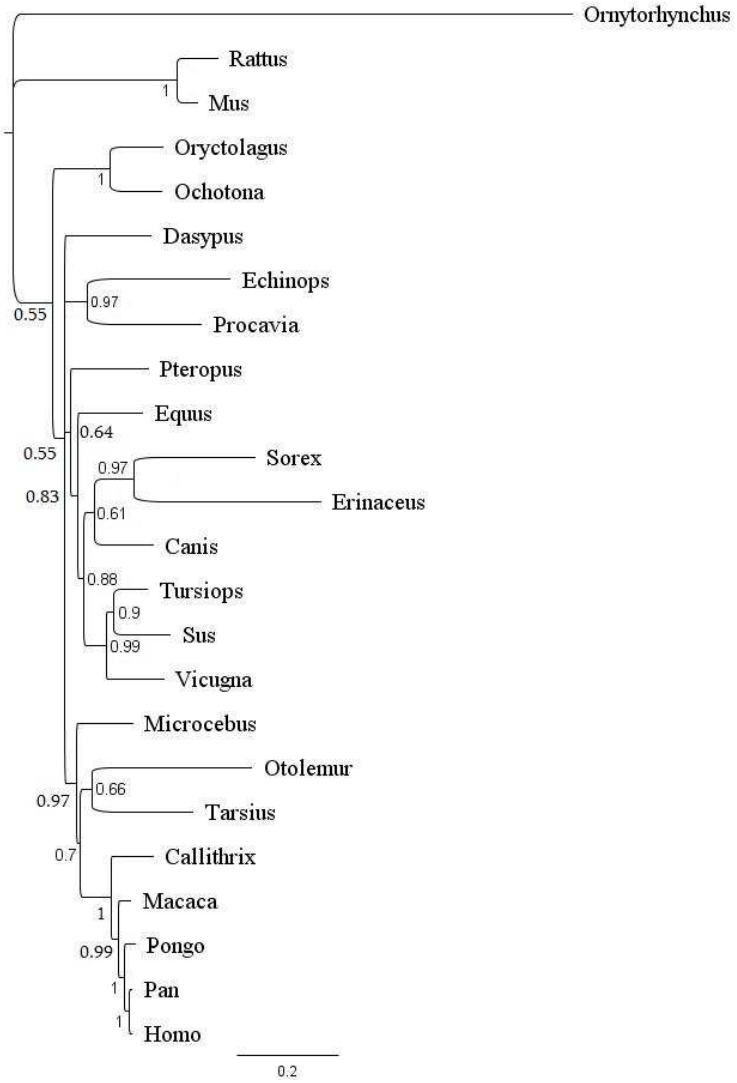


Figure A3.1.5. CSN2 Bayesian tree built for the mammalian dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

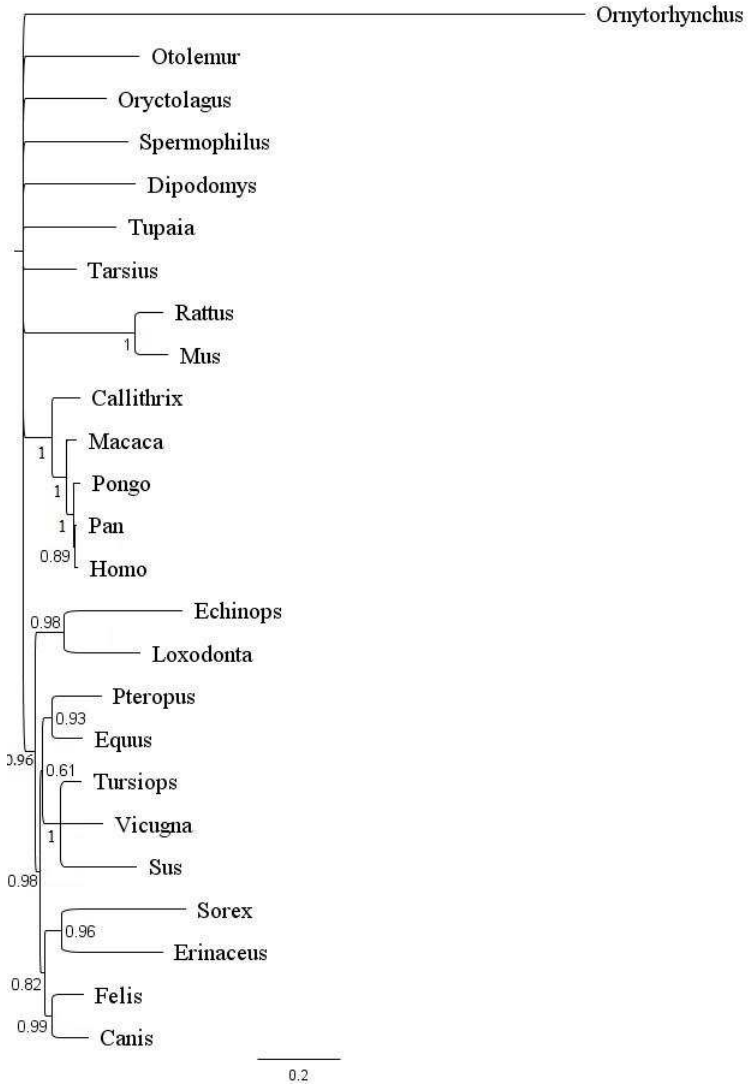


Figure A3.1.6. CSN3 Bayesian tree built for the mammalian dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

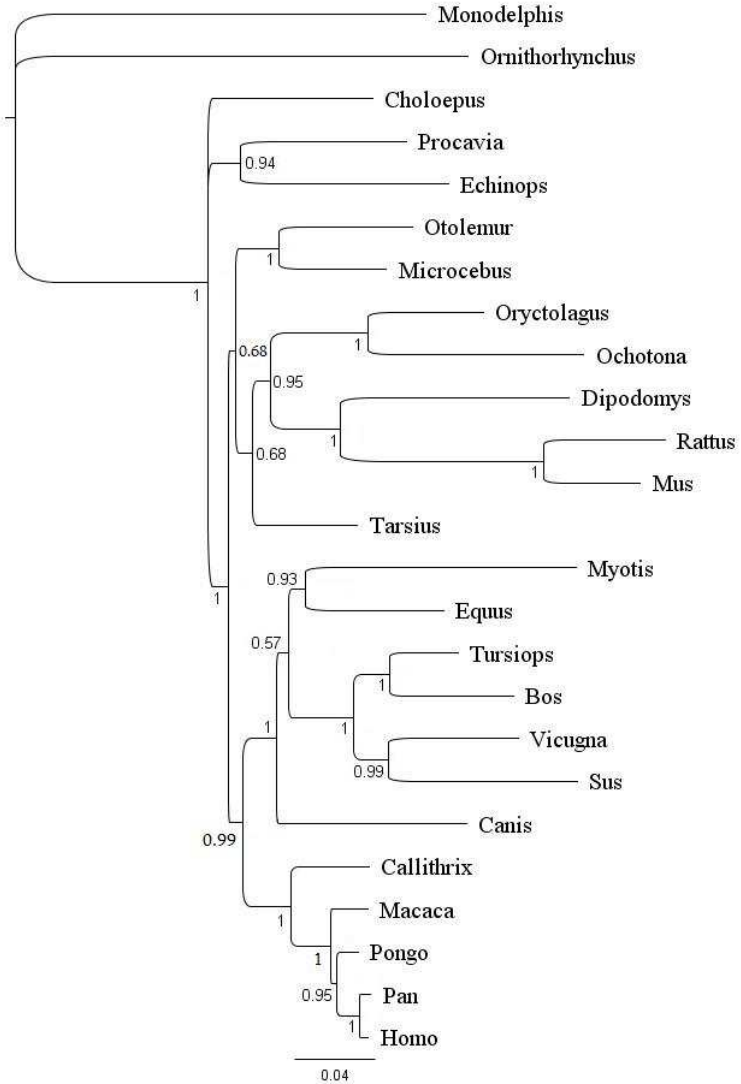


Figure A3.1.7. FGG Bayesian tree built for the mammalian dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

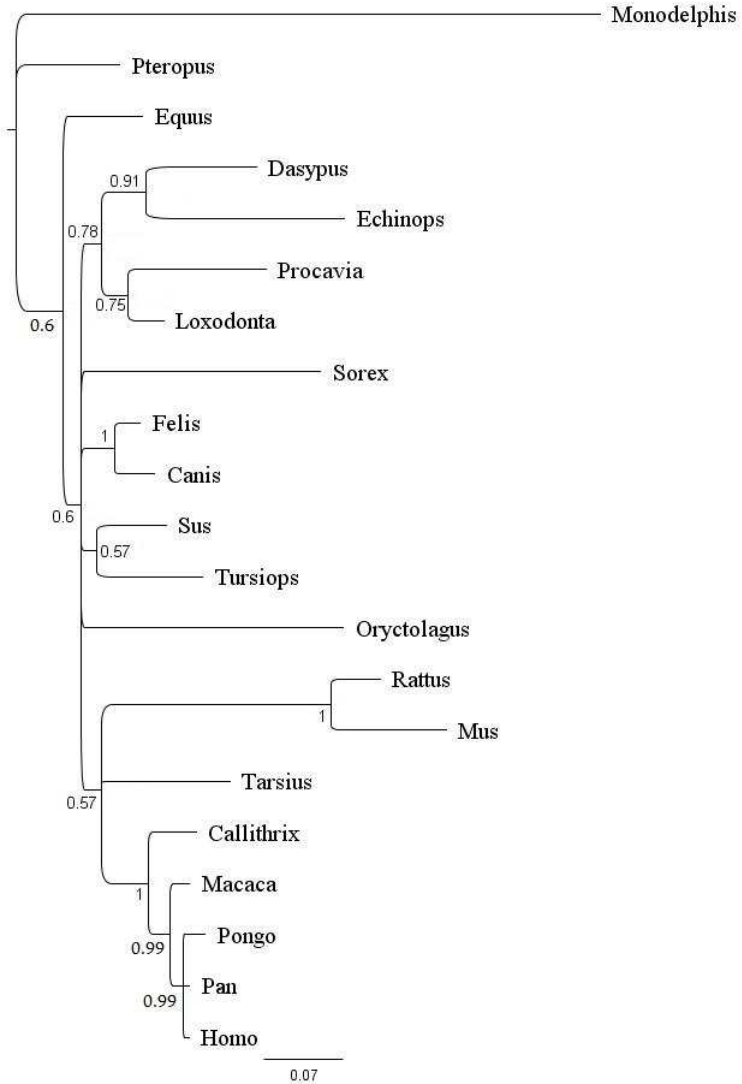


Figure A3.1.8. LALBA Bayesian tree built for the mammalian dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

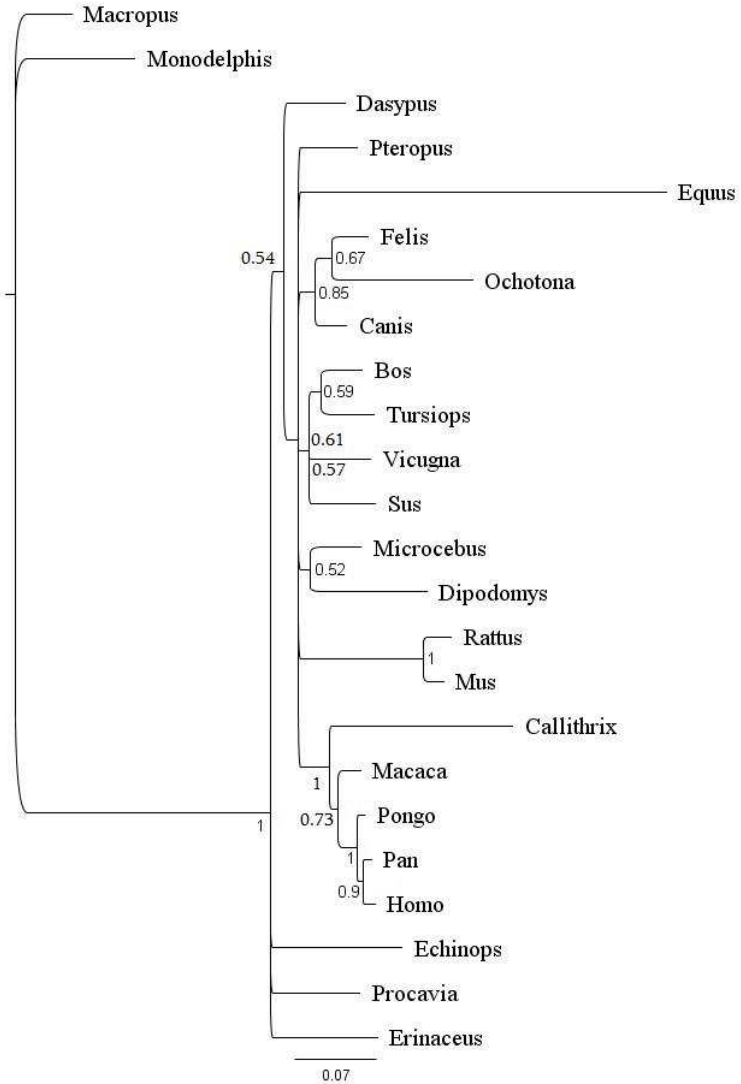


Figure A3.1.9. FIT1 Bayesian tree built for the mammalian dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

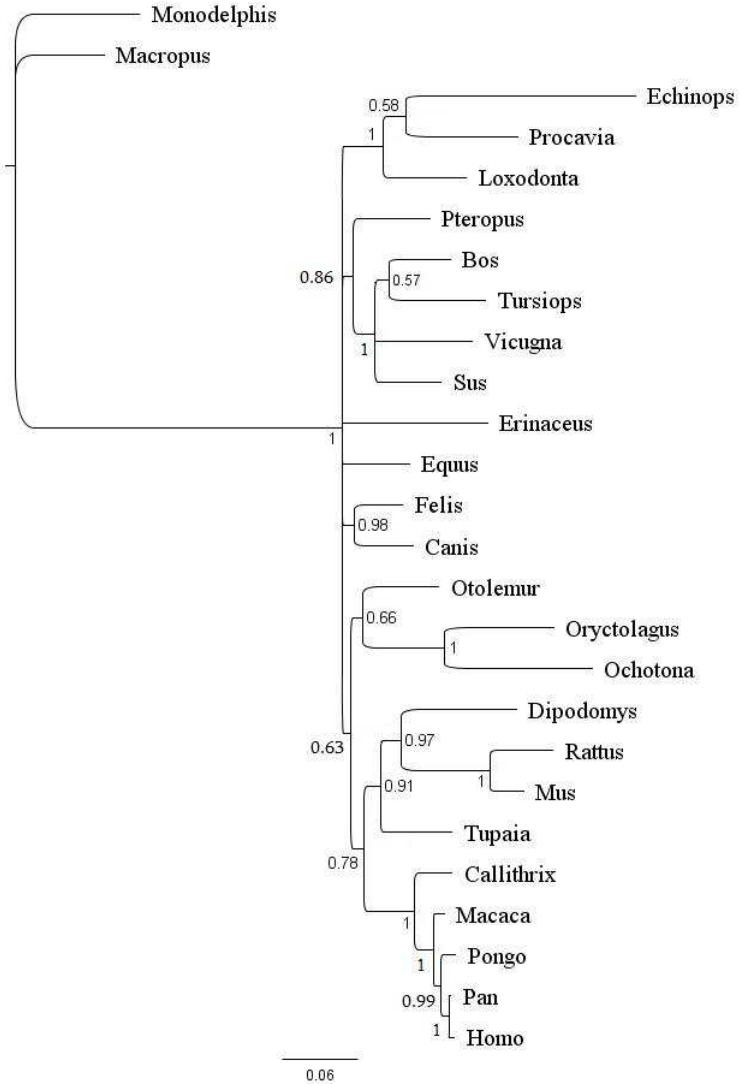


Figure A3.1.10. FIT2 Bayesian tree built for the mammalian dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

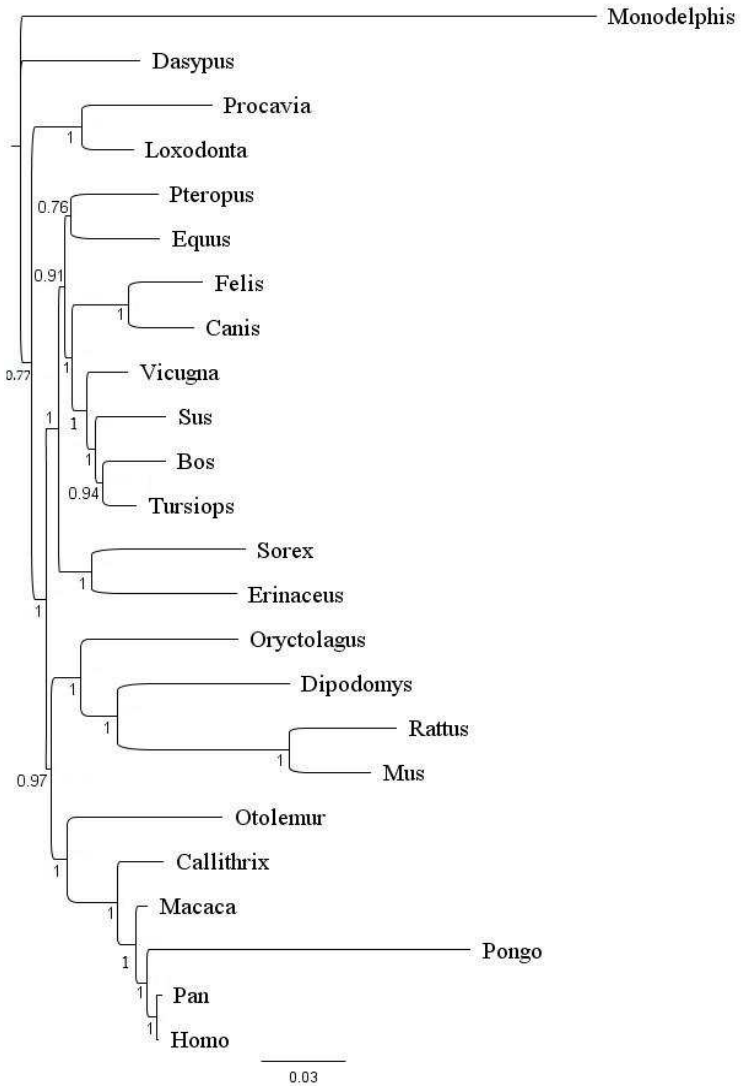


Figure A3.1.11. HIF1 Bayesian tree built for the mammalian dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

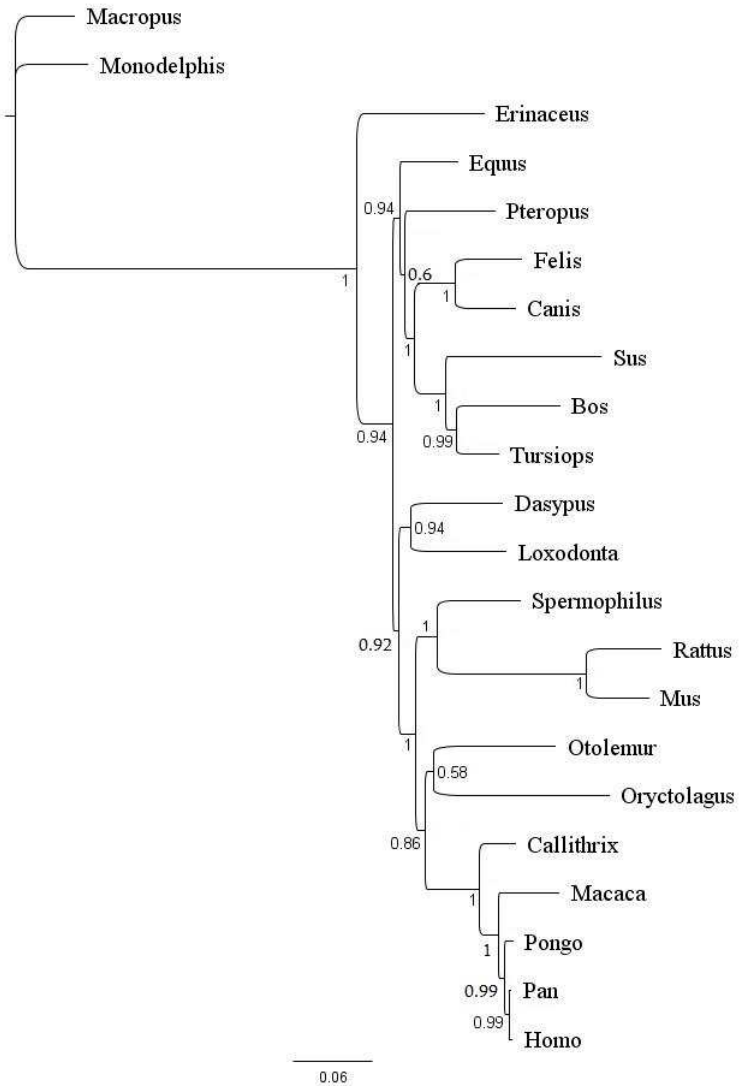


Figure A3.1.12. HIF2 Bayesian tree built for the mammalian dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

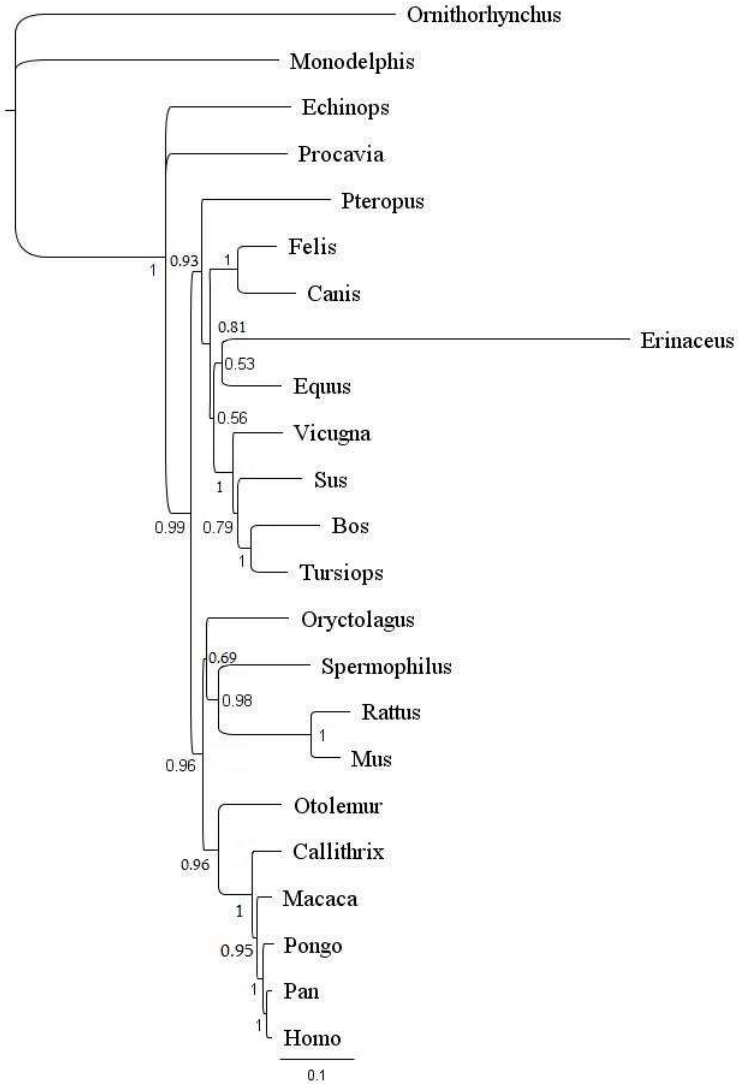


Figure A3.1.13. MYOC Bayesian tree built for the mammalian dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

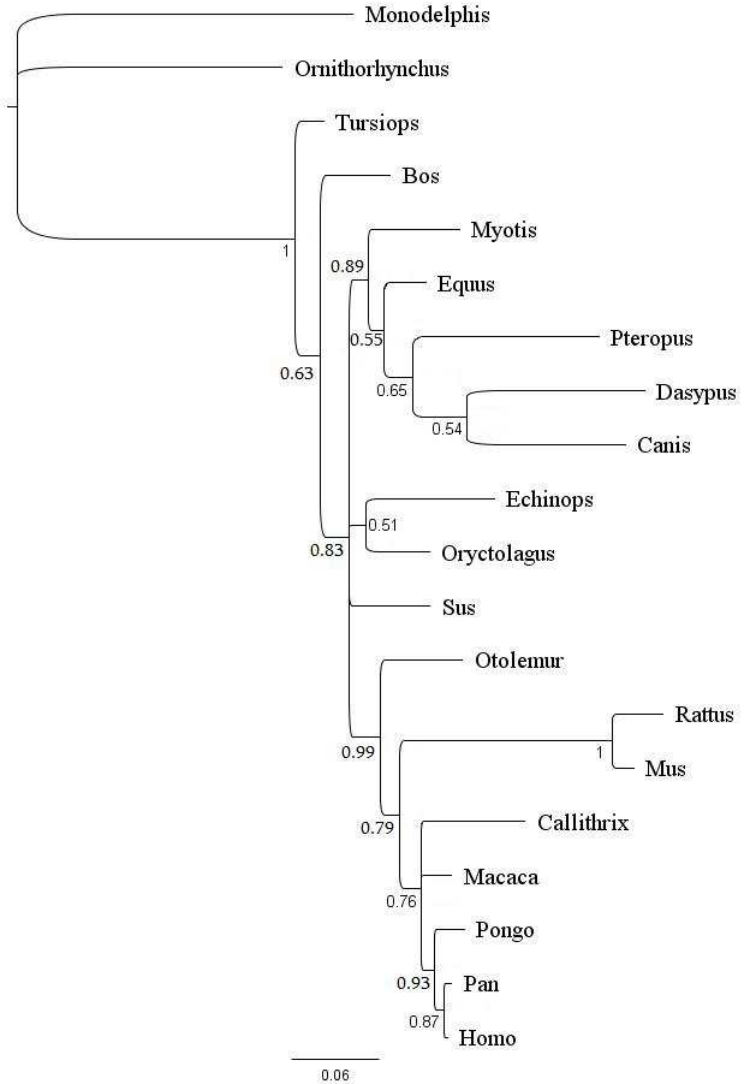


Figure A3.1.14. NGB Bayesian tree built for the mammalian dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

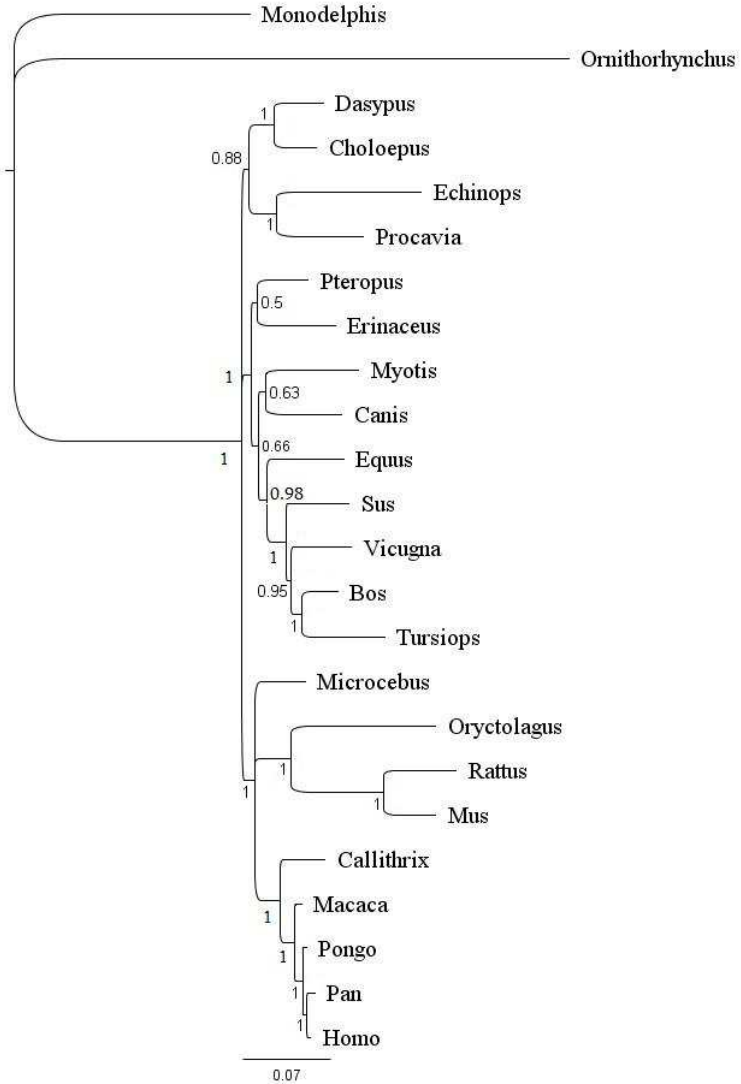


Figure A3.1.15. Prestin Bayesian tree built for the mammalian dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

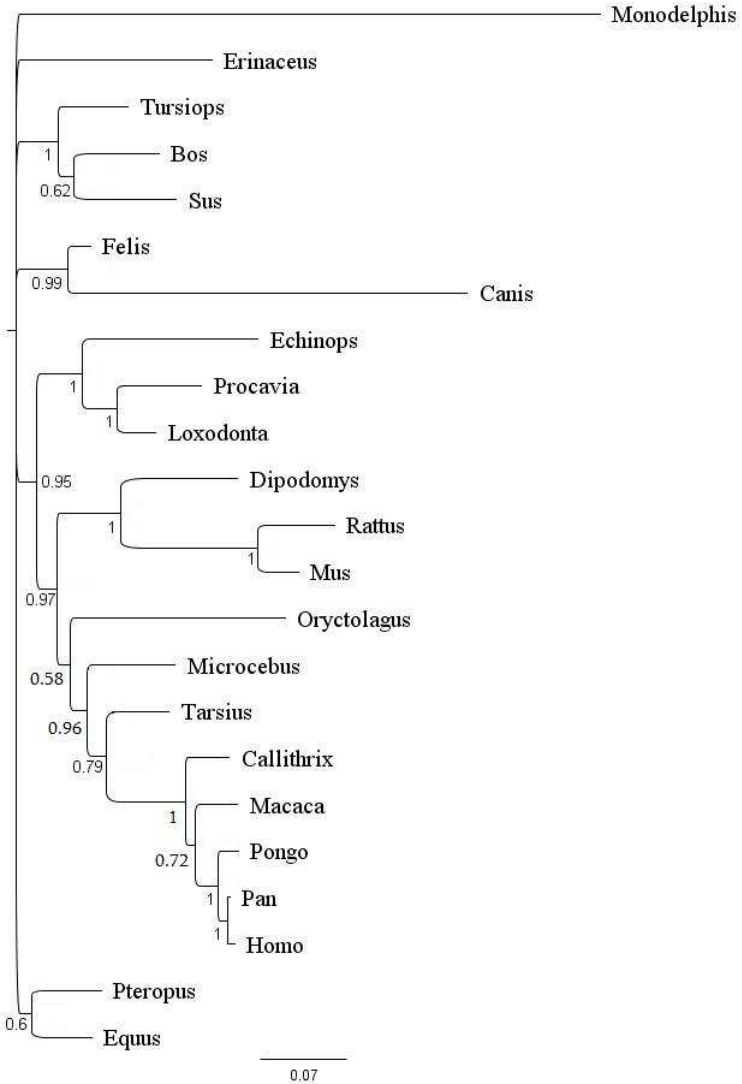


Figure A3.1.16. SP-C Bayesian tree built for the mammalian dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

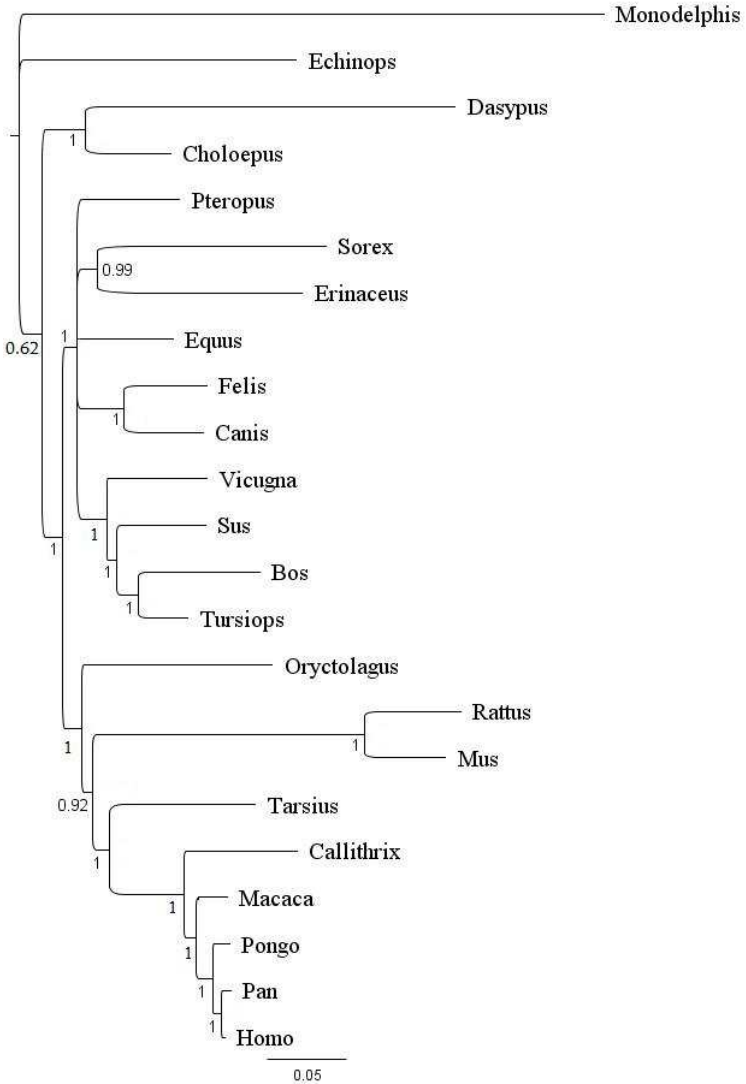


Figure A3.1.17. TLR3 Bayesian tree built for the mammalian dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

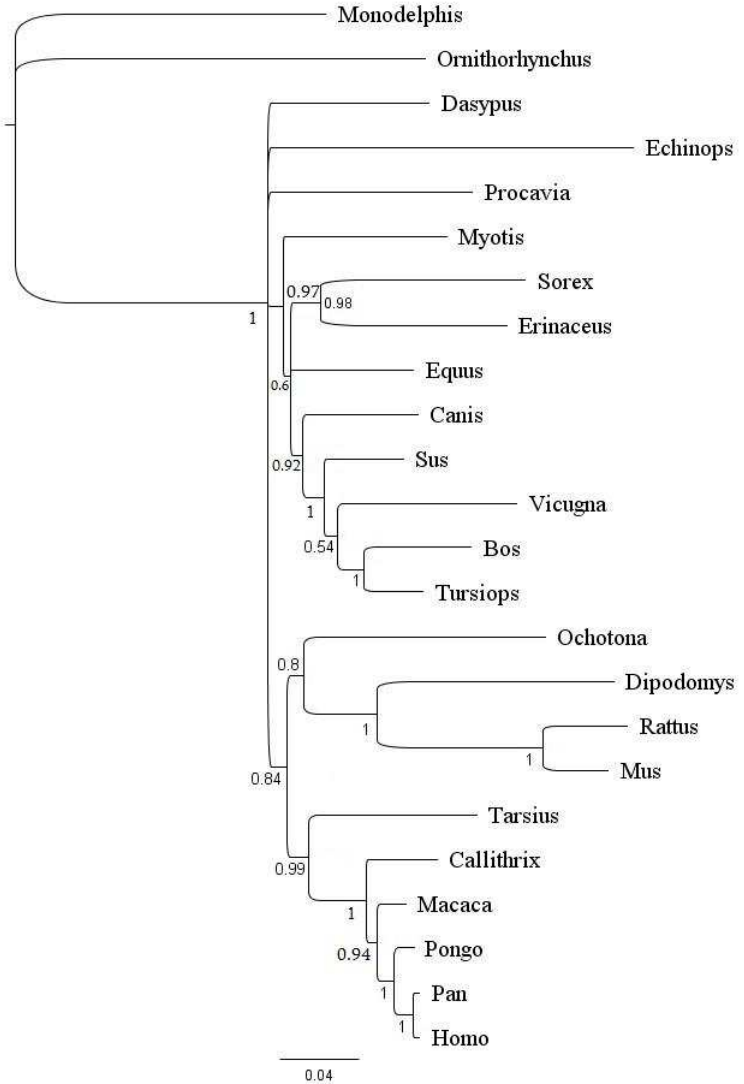


Figure A3.1.18. TYRP1 Bayesian tree built for the mammalian dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

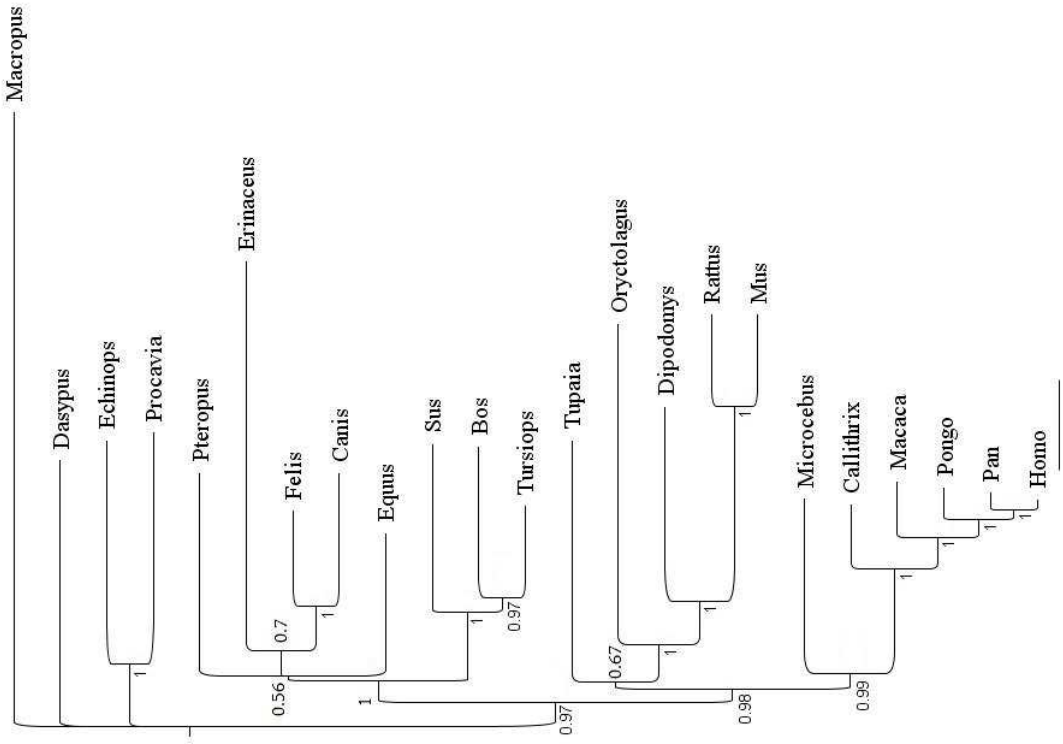


Figure A3.1.19, UT-A2 Bayesian tree built for the mammalian dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

Appendix 3.2 — Cetacean dataset dN/dS results and candidate markers phylogenetic trees

Table A3.2.1. dN/dS results for the several tested candidate markers in cetaceans. p represent the proportion of sites found to have the values of ω indicated. ω = dN/dS. PSS – list of codon positions found to be under selection.

Marker	Model	l	p0	p1	p2	ω0	ω1	ω2	p	q	Sig	PSS
CSN2	M0	-1861.63				1.407						--
	M3	-1847.63	0.683	0.009	0.308	0.652	3.514	3.514			<0.001	8T 80F 146P 299I 323P 413P 422A
	M1a	-1860.86	0.17	0.83		0	1					--
	M2a	-1847.63	0.683	0.317		0.652	1	3.514			<0.001	8T 80F 92L 146P 167I 197Q 260H 299I 323P 374F 413P 422A
	M7	-1864.28							1.168	0.005		--
	M8	-1847.62	0.712	0.288				3.65	1.232	0.484	<0.001	
CSN3	M0	-1229.33				1.22						--
	M3	-1224.37	0.745	0.031	0.223	0.637	3.49	3.49			<0.05	
	M1a	-1228.38	0.179	0.821		0	1					--
	M2a	-1224.29	0.156	0.167		0	1	4.152			<0.05	
	M7	-1228.4							0.02	0.005		--
	M8	-1224.29	0.826	0.174				4.044	0.025	0.005	<0.05	
FGG	M0	-2842.43				0.458						--
	M3	-2759.02	0.573	0.427	0	0.141	1.018	10.262			<0.001	
	M1a	-2759.03	0.57	0.43		0.139	1					--
	M2a	-2754.93	0.554	0.037		0.142	1	2.469			<0.05	104Y
	M7	-2755.8							0.428	0.533		--
	M8	-2750.52	0.874	0.126				1.622	0.543	0.921	<0.01	

Table A3.2.1 (cont.) — dN/dS results for the several tested candidate markers in cetaceans. p represent the proportion of sites found to have the values of ω indicated. ω = dN/dS. PSS – list of codon positions found to be under selection.

Marker	Model	l	p0	p1	p2	ω0	ω1	ω2	p	q	Sig	PSS
LALBA	M0	-1009.65				0.452						--
	M3	-1007.67	0.547	0.453	0	0.174	0.859	0.865			NS	
	M1a	-1007.69	0.652	0.348		0.224	1					--
	M2a	-1007.69	0.652	0.116		0.224	1	1			NS	
	M7	-1007.67							0.623	0.672		--
	M8	-1007.66	0.767	0.233				1	1.059	2.154	NS	
MC1R	M0	-2600.54				0.106						--
	M3	-2586.77	0.533	0.462	0.005	0.006	0.22	3.856			<0.001	
	M1a	-2593.35	0.949	0.051		0.083	1					--
	M2a	-2593.07	0.953	0.002		0.085	1	7.521			NS	
	M7	-2588.33							0.437	3.305		--
	M8	-2587.14	0.997	0.003				6.396	0.485	3.838	NS	
SP-C	M0	-852.04				0.248						--
	M3	-804.49	0.913	0.087	0	0.089	3.541	15.766			<0.001	
	M1a	-811.98	0.832	0.168		0.044	1					--
	M2a	-802.57	0.842	0.086		0.057	1	3.757			<0.001	5K 26L 29P
	M7	-813.93							0.123	0.438		--
	M8	-800.73	0.914	0.086				3.59	0.345	2.968	<0.001	

Table A3.2.1 (cont.) — dN/dS results for the several tested candidate markers in cetaceans. p represent the proportion of sites found to have the values of ω indicated. ω = dN/dS. PSS – list of codon positions found to be under selection.

Marker	Model	l	p0	p1	p2	ω0	ω1	ω2	p	q	Sig	PSS
UT-A2	M0	-7122.5				0.097						--
	M3	-7012.19	0.78	0.22	0	0.032					<0.001	
	M1a	-7041.13	0.926	0.074		0.067	1					--
	M2a	-7041.13	0.926	0		0.067	1	22.021			NS	
	M7	-7011.23							0.344	2.581		--
	M8	-7004.54	0.968	0.032				1	0.495	5.161	<0.01	
Prestin	M0	-17580.2				0.107						--
	M3	-17114.3	0.69	0.254	0.056	0.014	0.227	1.075			<0.001	50Y 116S 935H 1715F 1772A 1778M 1790T 1859D 1865I 1871Y 1889I 2099Q 2105Q 2129L 2174Q 2213A 2231S 2255A
	M1a	-17193.6	0.893	0.107		0.05	1					--
	M2a	-17188.4	0.892	0.006		0.051	1	3.883			<0.01	--
	M7	-17134.8							0.189	1.2		--
	M8	-17113.9	0.957	0.043				1.2	0.285	3.103	<0.001	
Protamine	M0	-889.99				1.208						--
	M3	-847.57	0.779	0.148	0.073	0.672	5.196	24.227			<0.001	65R 140A 143I
	M1a	-879.76	0.287	0.713		0.046	1					--
	M2a	-847.96	0.187	0.13		0	1	15.031			<0.001	65R 74R 140A 143I
	M7	-879.55							0.047	0.017		--
	M8	-847.97	0.869	0.131				15.131	0.02	0.005	<0.001	

Table A3.2.1 (cont.) — dN/dS results for the several tested candidate markers in cetaceans. p represent the proportion of sites found to have the values of ω indicated. ω = dN/dS. PSS – list of codon positions found to be under selection.

Marker	Model	l	p0	p1	p2	ω0	ω1	ω2	p	q	Sig	PSS
ZP3	M0	-584.03				0.631						--
	M3	-570.33	0.016	0.868	0.115	0.265	0.265	5.126			<0.001	146S 167K 179S
	M1a	-575.85	0.607	0.393		0.054	1					--
	M2a	-570.33	0.885	0.115		0.265	1	5.126			<0.01	146S 167K 179S
	M7	-577.73							0.02	0.025		--
	M8	-570.33	0.885	0.115				5.137	36.003	99	<0.001	

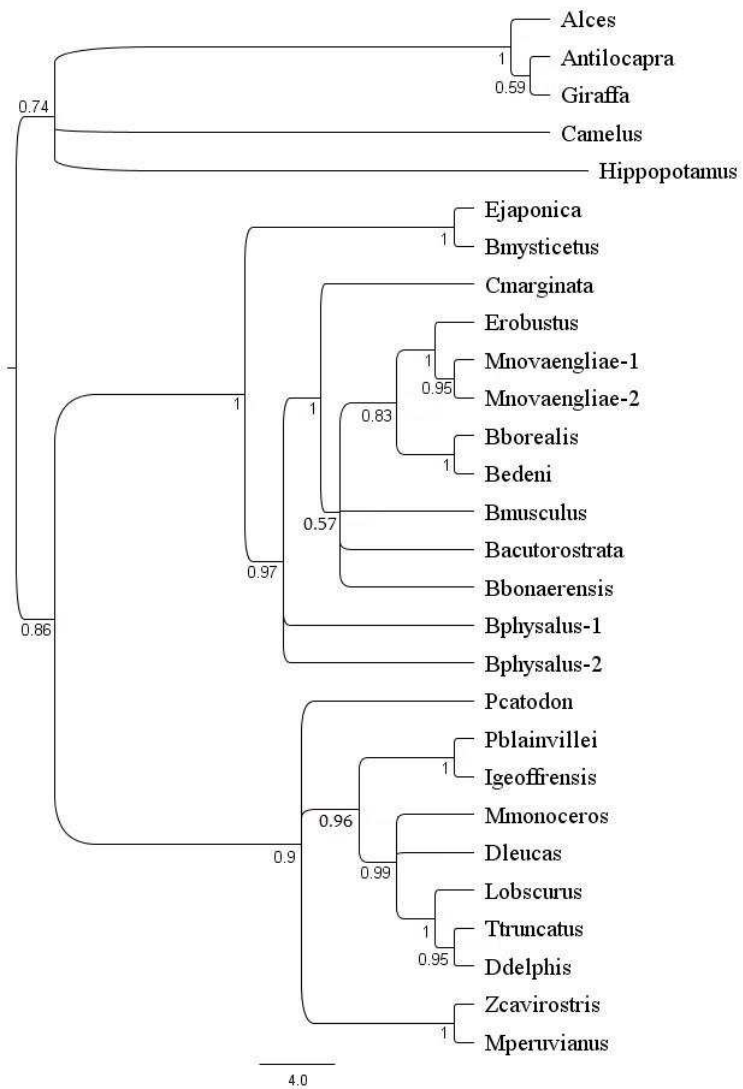


Figure A3.2.1. CSN2 Bayesian tree built for the cetacean dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

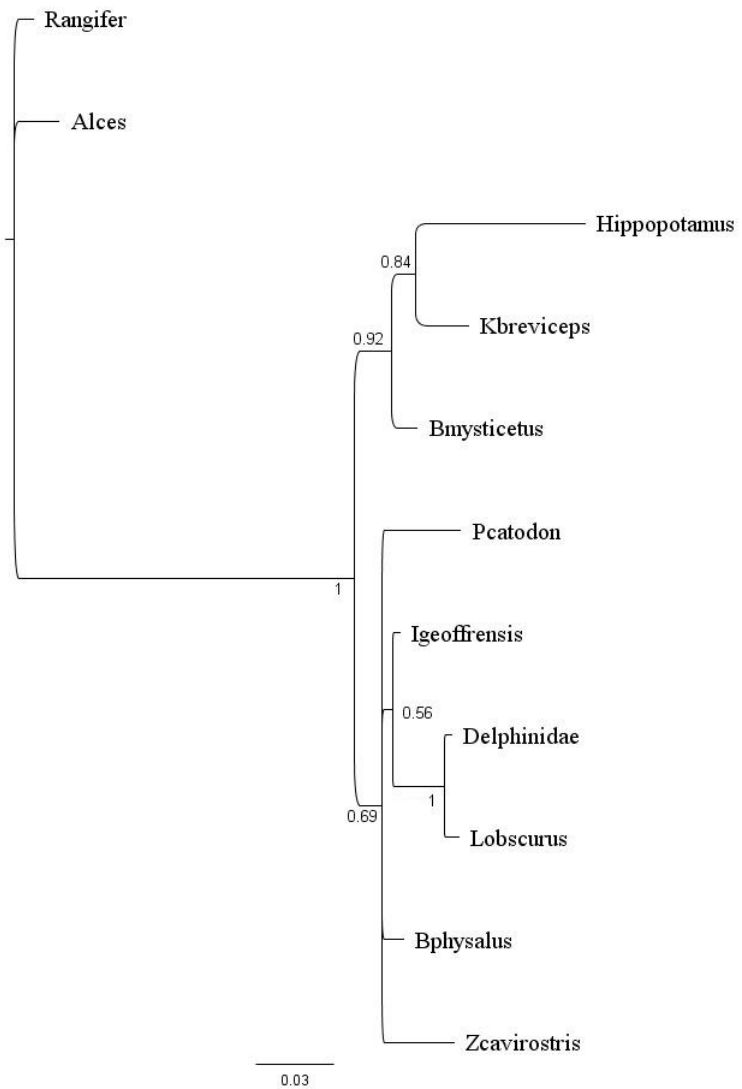


Figure A3.2.2. CSN3 Bayesian tree built for the cetacean dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

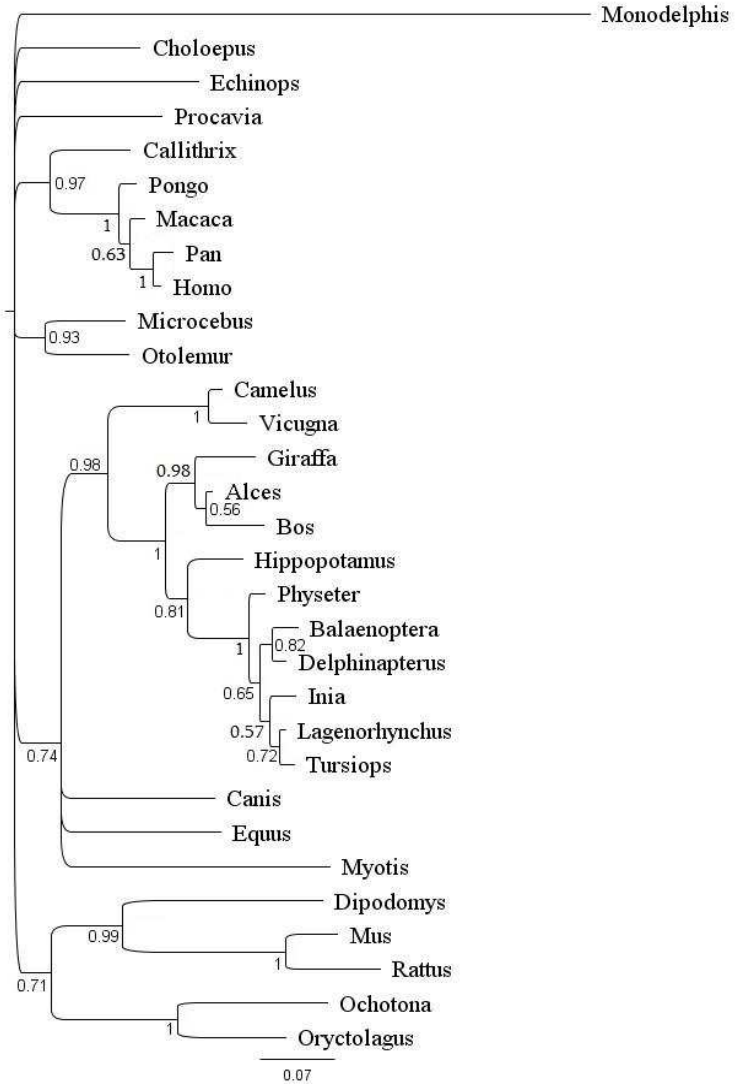


Figure A3.2.3. FGG Bayesian tree built for the cetacean dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

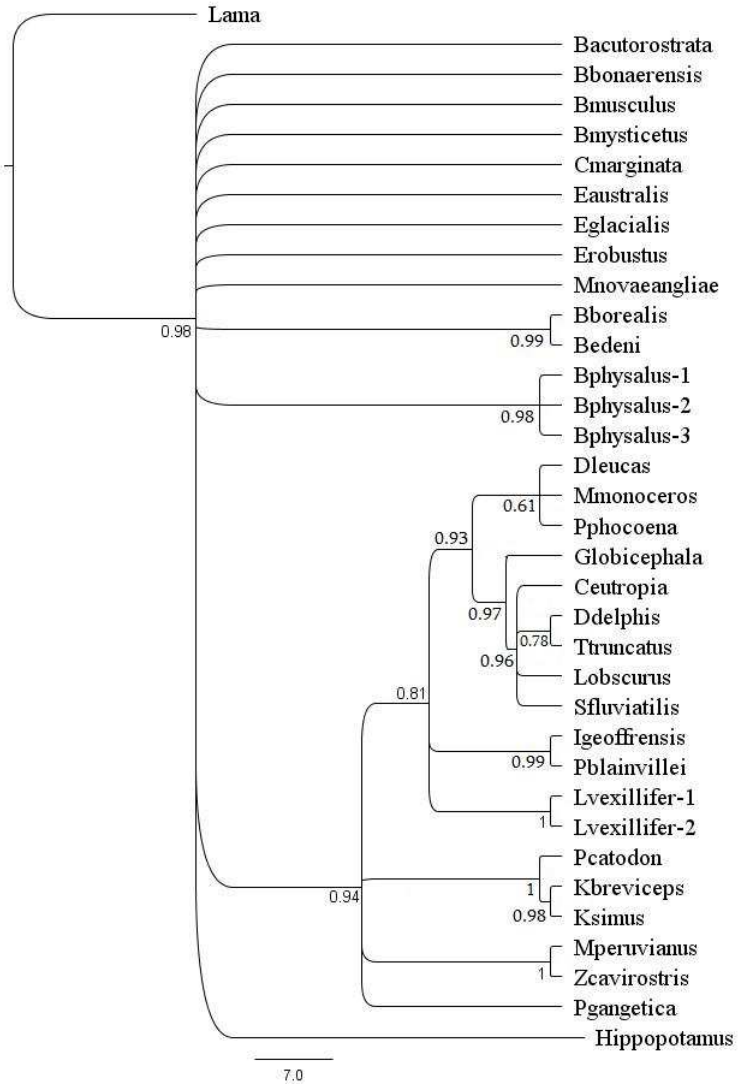


Figure A3.2.4. LALBA Bayesian tree built for the cetacean dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

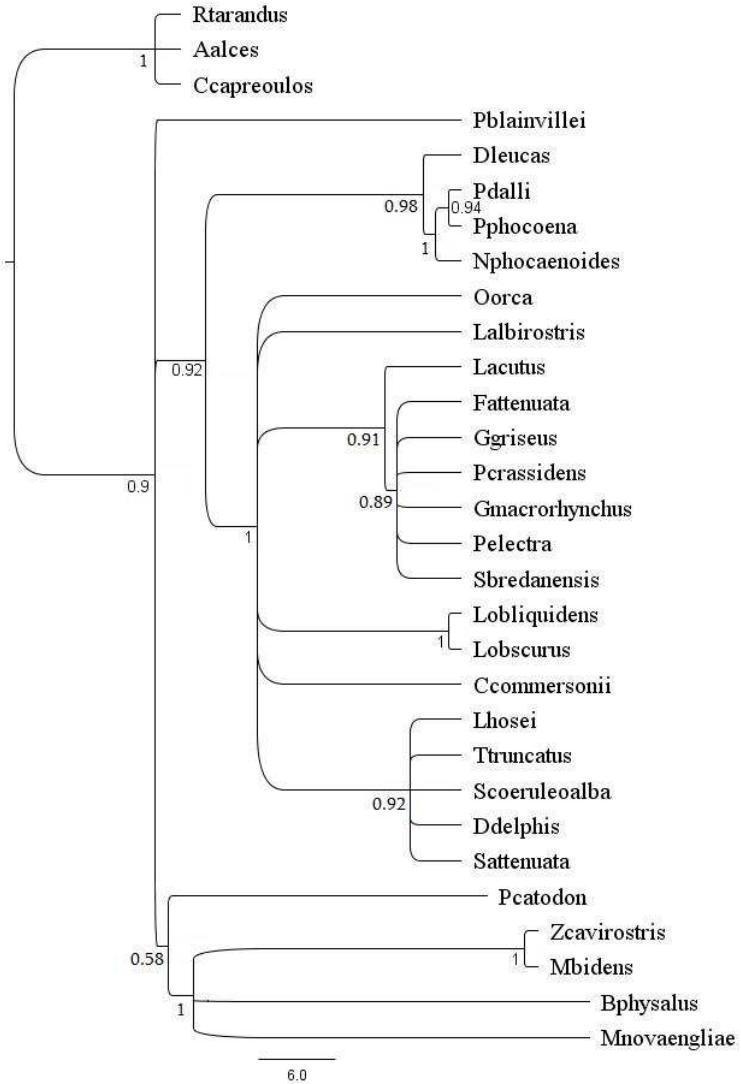


Figure A3.2.5. MC1R Bayesian tree built for the cetacean dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

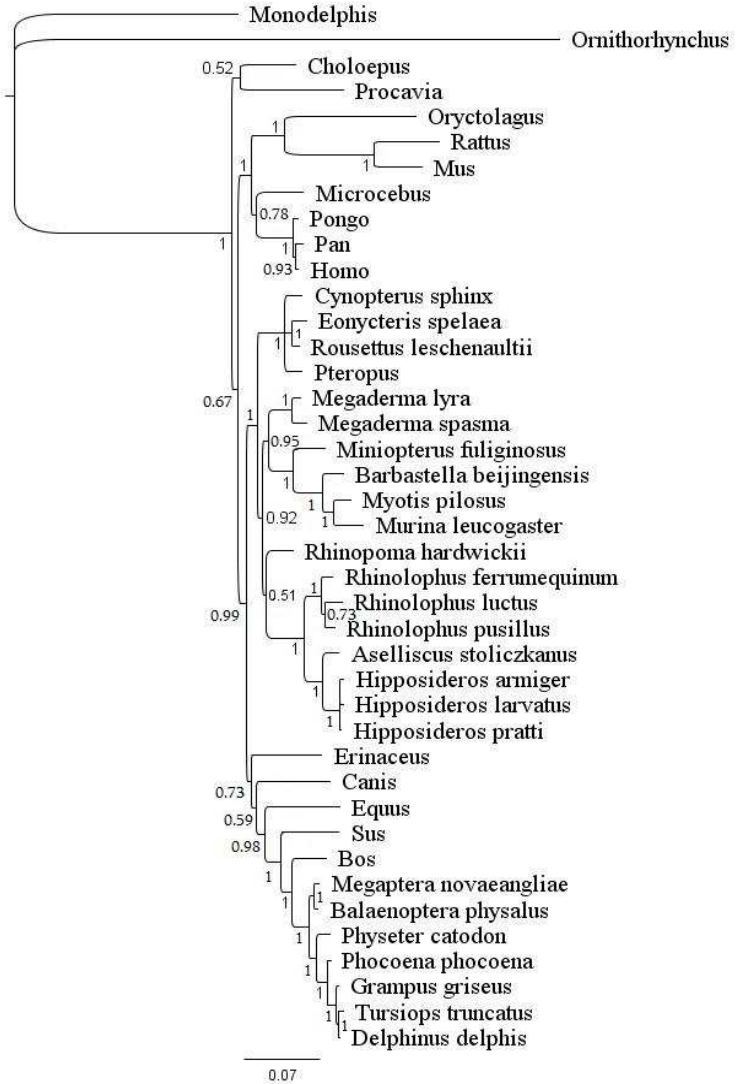


Figure A3.2.6. Prestin Bayesian tree built for the cetacean dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

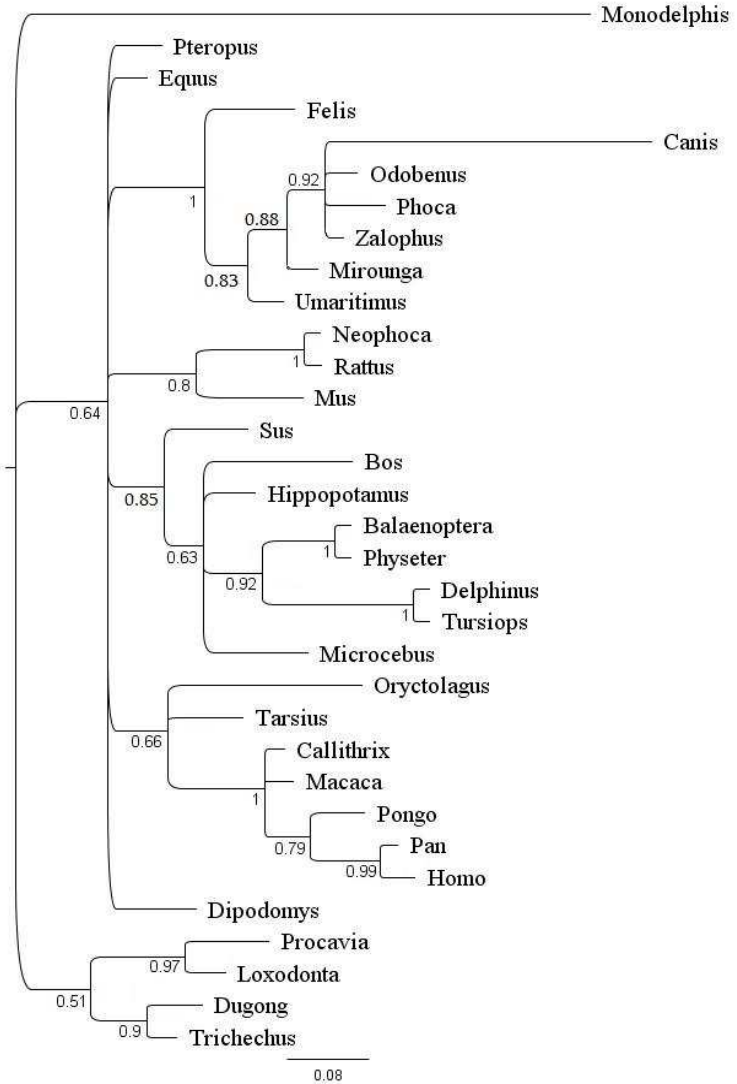


Figure A3.2.7. SP-C Bayesian tree built for the cetacean dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

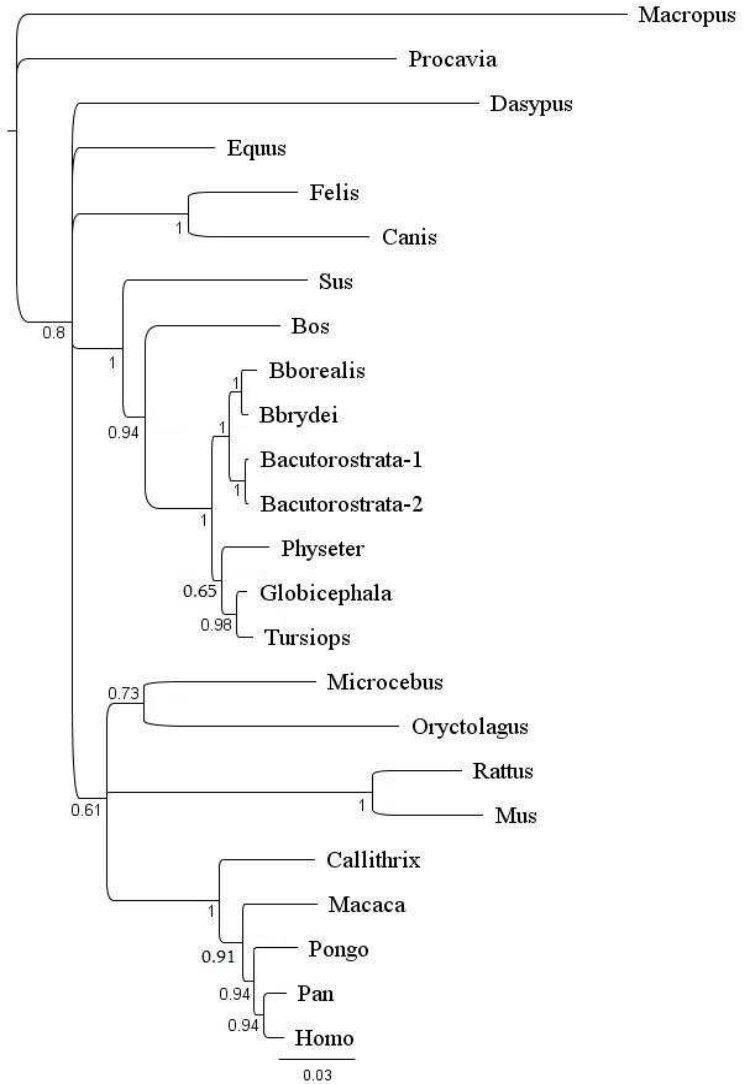


Figure A3.2.8. UT-A2 Bayesian tree built for the cetacean dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

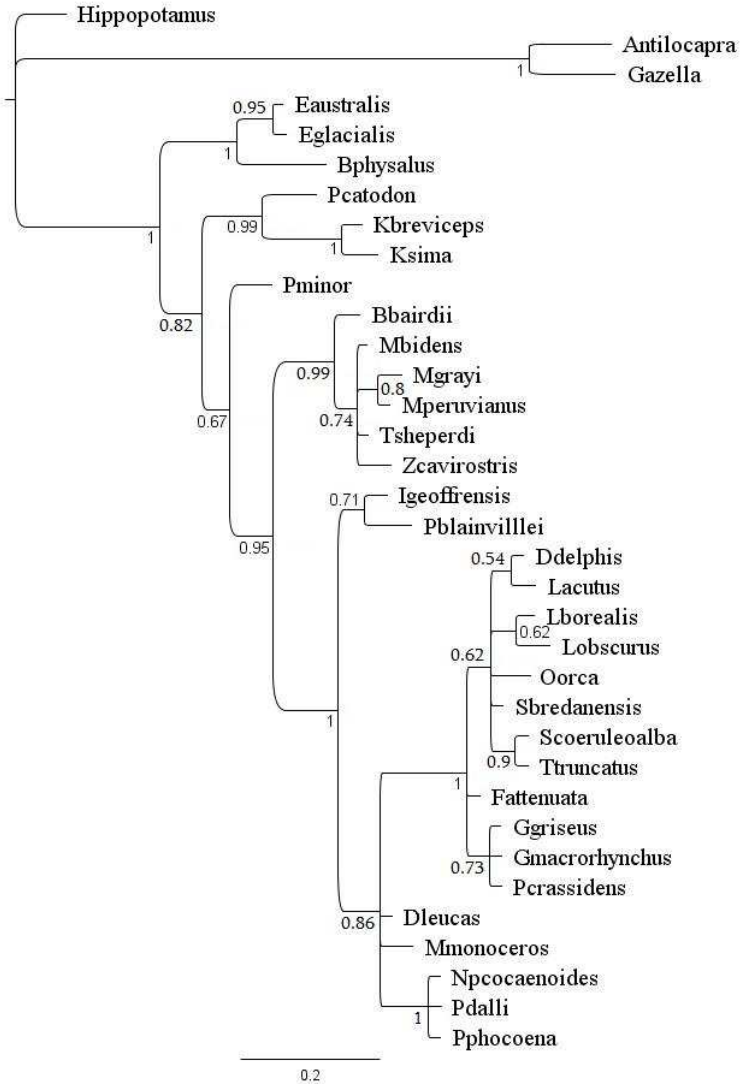


Figure A3.2.9. Protamine Bayesian tree built for the cetacean dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

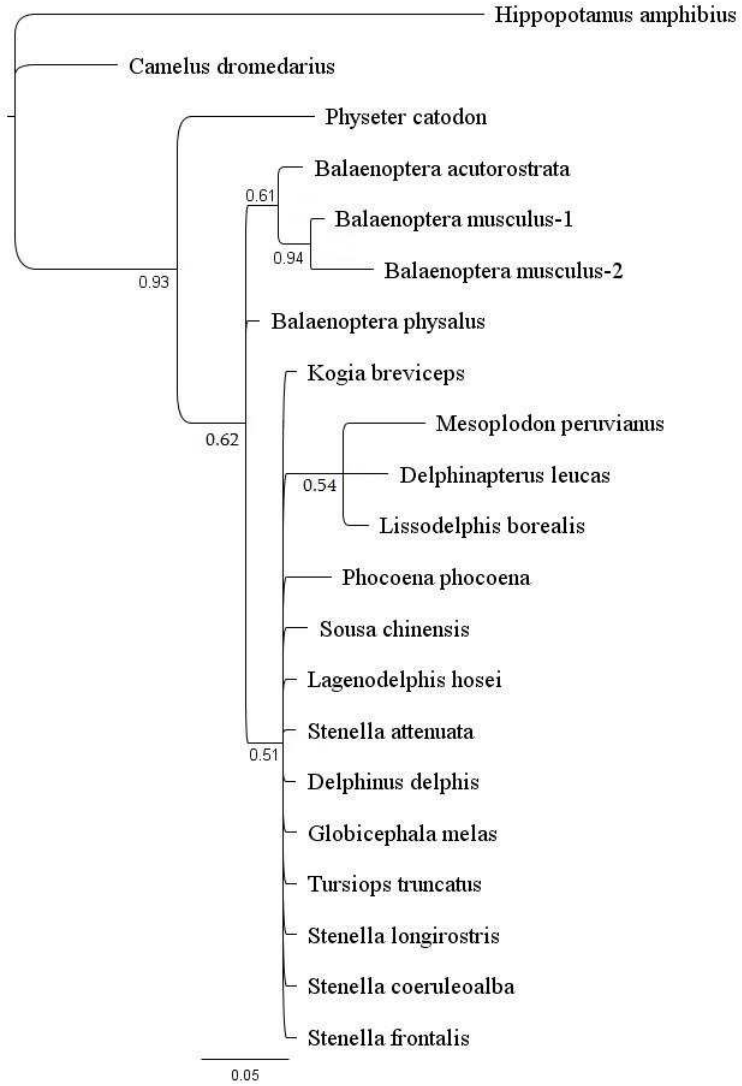
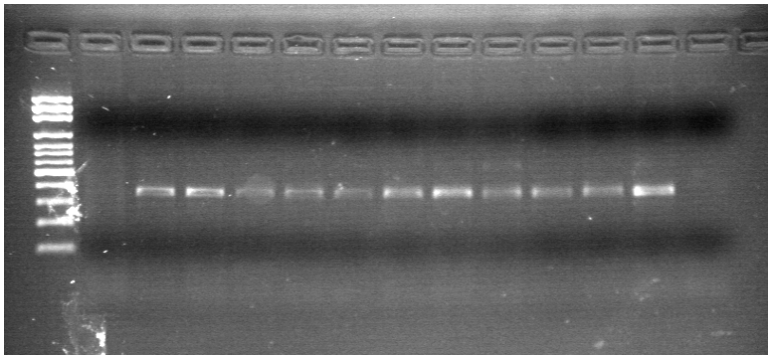
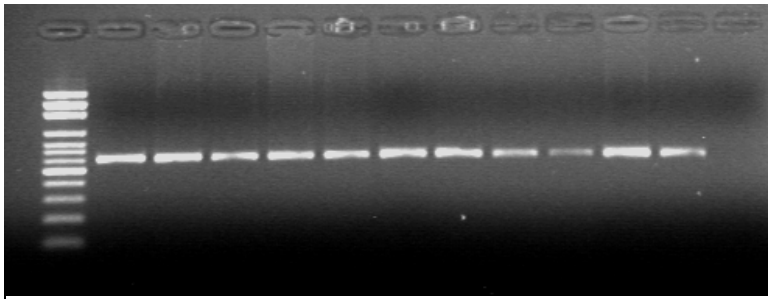


Figure A3.2.10. ZP3 Bayesian tree built for the cetacean dataset. Numbers show node posterior probability. Picture drawn using GENEIOUS

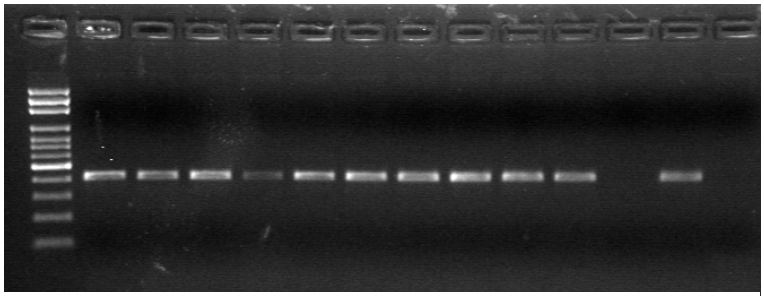
Appendix 3.3 — Agarose gel pictures from successfully amplified nuclear markers



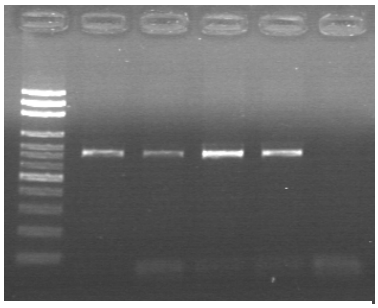
Protamine 1



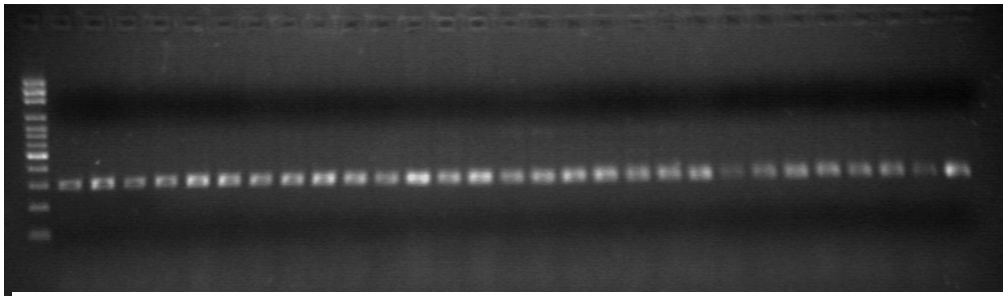
Aquaporin 1 (AQP1)



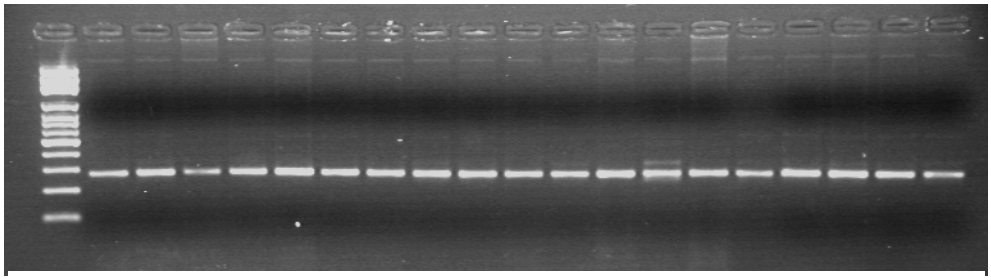
β-casein (CSN2)



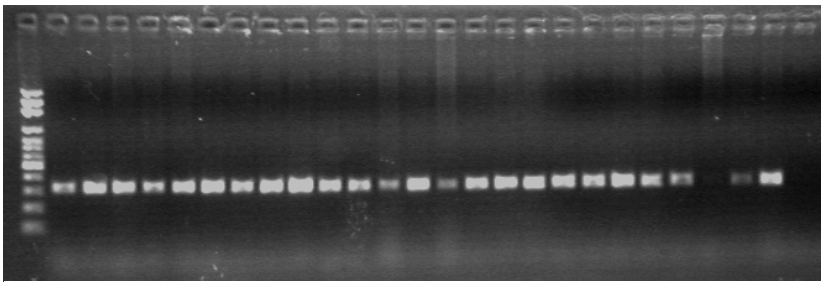
Myocilin (MYOC)



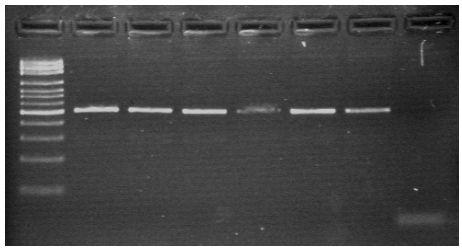
Lung surfactant pTyrosinaserotein C (SP-C)



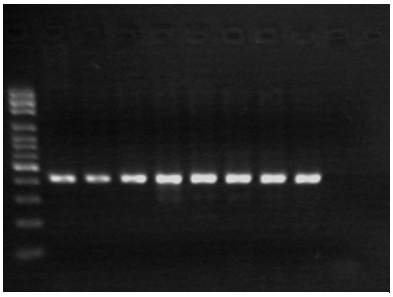
Urea-transporter 2, α chain (UT-A2)



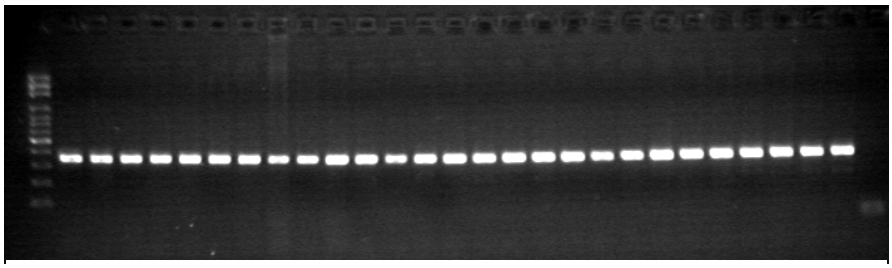
Tyrosinase-related protein 1 (TYRP1), exon 1



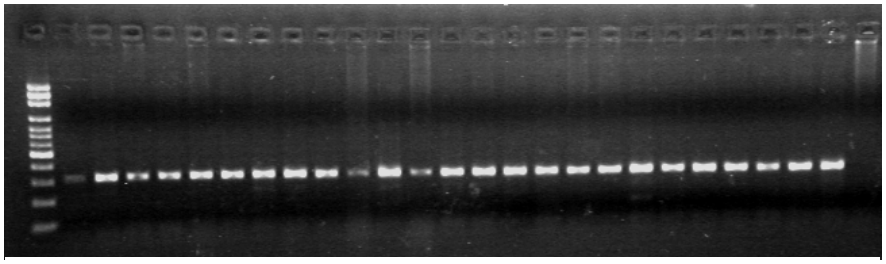
TYRP1 exon 2



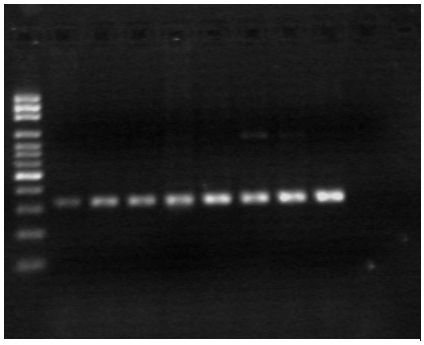
TYRP1 exon 3



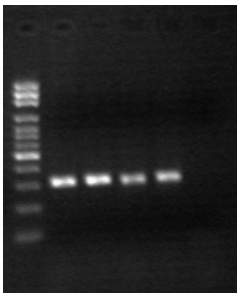
TYRP1 exon 4



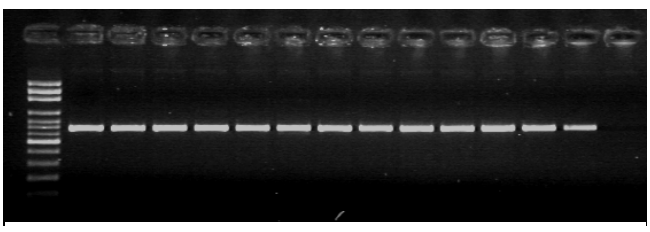
TYRP1 exon 5



TYRP1 exon 6



TYRP1 exon 7



TYRP1 exon 8

Appendix 4 — List of Abbreviations

ABC - Approximate Bayesian Computation

AFLP - Amplified Fragment Length Polymorphism

AMOVA – Analysis of Molecular Variance

BSEA – Black Sea

bp – Base pairs

CA - Correspondence Analysis

CAMKA2 - Ca/calmodulin-dependent protein kinase II, α chain

AQP1 - Aquaporin 1

AQP6 - Aquaporin 6

ASIP – Agouti Signalling Protein

CI – Confidence Interval

CSN2 - α-Casein

CSN3 - κ-Casein

df – Degrees of Freedom

DNA - Deoxyribonucleic Acid

dN/dS – Proportion between non-synonymous and synonymous mutations

dNTP - Deoxyribonucleotide triphosphate

EMED – Eastern Mediterraean

FAM – Carboxyfluorescein

FCA - Factorial Correspondence Analysis

FGG - γ-Fibrinogen

FIT - Fat Inducing Transcript

GC – Gulf of California

HEX - Hexachlorofluorescein phosphoramidite

HIF - Hypoxia Inducible Factor

IAM - Infinite Allele Mutation model

ICNB - Instituto para a Conservação da Natureza e Biodiversidade

IP – Indo Pacific

IUCN - International Union for Conservation of Nature

kyrs – Thousand years

kyrsBP - Thousand years before present

LALBA - α-Lactalbumin

LGM – Last Glacial Maxima

MC1R - Melanocortin Receptor 1

MHC - Major Histocompatibility Complex

MSH – Melanocyte Stimulating Hormone

mtDNA – Mitochondrial DNA

MYOC - Myocilin

Myrs – Million years

MyrsBP – Million years before present

Ne – Effective Population Size

NGB - Neuroglobin

OAG – Open Angle Glaucoma

PCA - Principal Component Analysis

PCR – Polymerase Chain Reaction

QTL – Quantitative Trait Loci

SA – South Africa

SABD – South Australian Bottlenose Dolphin

SCO – Scotland

SMM - Stepwise Mutation Model

SNP – Single Nucleotide Polymorphism

SP-C - Lung surfactant protein C

SSCP – Single Strand Conformation Polymorphism

SST – Sea Surface Temperature

Ta – *Tursiops aduncus*

TBE – Tris-Borate-EDTA buffer

TE – Tris-EDTA buffer

TLR - Toll-like receptor

TMRCA – Time to the Most Recent Common Ancestor

TPM – Two Phased Model

Tt – *Tursiops truncatus*

TYRP1 - Tyrosinase-related protein 1

UT-A2 - Urea-transporter 2, α chain

WNAC – Western North Atlantic Coastal

WNAP – Western North Atlantic Pelagic

ZP – Zona Pellucida

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